

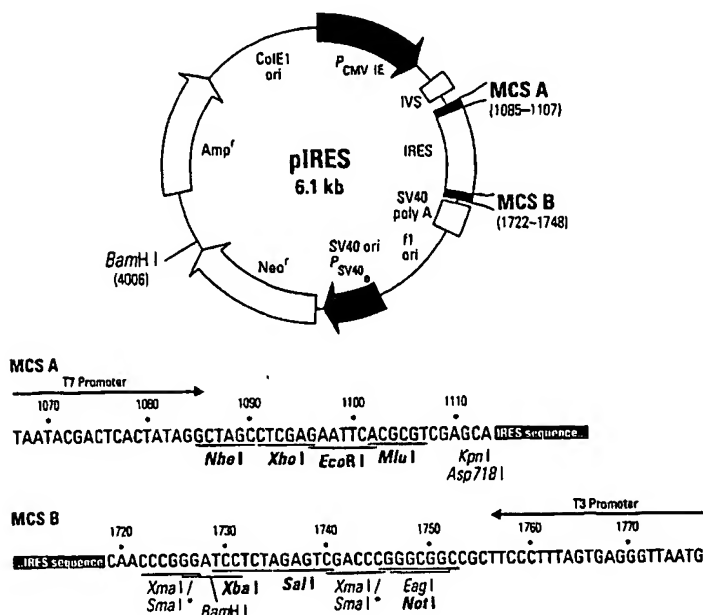
(19) World Intellectual Property Organization
International Bureau(43) International Publication Date
21 June 2001 (21.06.2001)

PCT

(10) International Publication Number
WO 01/44296 A1

- (51) International Patent Classification⁷: **C07K 14/705**
- (21) International Application Number: **PCT/GB00/04828**
- (22) International Filing Date:
15 December 2000 (15.12.2000)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
9929993.5 17 December 1999 (17.12.1999) GB
- (71) Applicant (for all designated States except US): **AVIDEX LIMITED** [GB/GB]; 57 C Milton Park, Abingdon, Oxfordshire OX14 4RX (GB).
- (72) Inventor; and
- (75) Inventor/Applicant (for US only): **JAKOBSEN, Bent, Karsten** [DK/GB]; 57 C Milton Park, Abingdon, Oxfordshire OX14 4RX (GB).
- (54) Agents: **LEE, Nicholas, John** et al.; Kilburn & Strode, 20 Red Lion Street, London WC1R 4PJ (GB).
- (81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- Published:
— With international search report.
— Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.

[Continued on next page]

(54) Title: METHODS OF INHIBITING THE BINDING OF A CD8⁺ T CELL TO A CLASS I MHC EMPLOYING A MODIFIED BETA 2 MICROGLOBULIN

(57) Abstract: The present invention provides a method of inhibiting the binding of a CD8⁺ T cell to a class I Major Histocompatibility Complex (MHC). The method comprises exposing the class I MHC to a modified β_2 -microglobulin whose binding to CD8 is inhibited. Also provided are modified β_2 -microglobulin molecules and nucleic acids encoding such molecules.

WO 01/44296 A1



For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

METHODS OF INHIBITING THE BINDING OF A CD8+ T CELL TO A CLASS I MHC EMPLOYING
A MODIFIED BETA 2 MICROGLOBULIN

The present invention relates to modified β_2 -microglobulin (β_2m) proteins and in particular to their use in immunosuppressive therapy, in particular as inhibitors of
5 cytotoxic T cell responses.

β_2 -microglobulin forms a part of the Class I Major Histocompatibility Complex (MHC). MHC molecules are specialised protein complexes which present short protein fragments (peptide antigens) on the cell surface for recognition by the
10 cellular arm of the adaptive immune system.

Class I MHC is a dimeric protein complex consisting of a variable heavy chain and a constant light chain, β_2 -microglobulin. Class I MHC presents peptides which are processed intracellularly, loaded into a binding cleft in the MHC, and transported to
15 the cell surface where the complex is anchored in the membrane by the MHC heavy chain. Peptides are usually 8-11 amino acids in length, depending on the degree of arching introduced in the peptide when bound in the MHC. The binding cleft which is formed by the membrane distal $\alpha 1$ and $\alpha 2$ domains of the MHC heavy chain has "closed" ends, imposing quite tight restrictions on the length of peptide which can
20 be bound.

β_2 -microglobulin is a polypeptide which is found free in serum, which is non-covalently associated with MHC Class I molecules at the cell surface and which can exchange in the MHC complex with other free β_2m molecules (Bernabeu, *et al.*
25 *Nature* 308: 642-5 (1984); Cook, *et al. J Immunol* 157: 2256-61 (1996); Horig, *et al. Proc Natl Acad Sci U S A* 94: 13826-31 (1997); Hyafil & Strominger, *Proc Natl Acad Sci U S A* 76: 5834-8 (1979); Luscher, *et al. J Immunol* 153: 5068-81 (1994); Parker, *et al. J Immunol* 149: 1896-904 (1992); Smith, *et al. Proc Natl Acad Sci U S*

A 89: 7767-71 (1992)). Unlike MHC heavy chain polypeptides, β_2m is not anchored in the cell membrane. β_2m has the structure of an immunoglobulin C (constant) domain and also associates with a number of other class I - related structures, such as the products of the CD1 genes in man.

5

A wide spectrum of cells can present antigen, as MHC-peptide, and the cells which have that property are known as antigen presenting cells (APCs). The type of cell which presents a particular antigen depends upon how and where the antigen first encounters cells of the immune system. APCs include the interdigitating dendritic cells found in the T cell areas of the lymph nodes and spleen in large numbers; Langerhan's cells in the skin; follicular dendritic cells in B cell areas of the lymphoid tissue; monocytes, macrophages and other cells of the monocyte/macrophage lineage; B cells and T cells; and a variety of other cells such as endothelial cells and fibroblasts which are not classical APCs but can act in the manner of an APC.

15

APCs are recognised by a subgroup of lymphocytes which mature in the thymus (T cells) where they undergo a selection procedure ensuring that T cells which respond to self-peptides are eradicated (negative selection). In addition, T cells which do not have the ability to recognise the MHC variants which are presented (in man the HLA haplotypes) fail to mature (positive selection).

20

Recognition of specific MHC-peptide complexes by T cells is mediated by the T cell receptor (TCR) which consists of an α - and a β chain, both of which are anchored in the membrane. In a recombination process similar to that observed for antibody genes, the TCR α - and β - genes rearrange from Variable, Joining, Diversity and Constant elements creating enormous diversity in the extracellular antigen binding domains (10^{13} to 10^{15} different possibilities).

25

Antibody receptors and TCRs are the only two types of molecules which recognise antigens in a specific manner, and thus the TCR is the only receptor specific for particular peptide antigens presented in MHC, the alien peptide often being the only sign of an abnormality within a cell.

5

The vast majority of T cells restricted by Class I MHC-peptide complexes also require the engagement of the coreceptor CD8 for activation, while T cells restricted by Class II MHC require the engagement of CD4. The exact function of the coreceptors in T cell activation is not yet entirely clarified; neither are the critical mechanisms and parameters controlling activation. However, both CD8 and CD4 have cytoplasmic domains which are associated with the kinase $p56^{lck}$ which is involved in the very earliest tyrosine phosphorylation events which characterise T cell activation. CD8 is a dimeric receptor, expressed either in an $\alpha\alpha$ form or, more commonly, in an $\alpha\beta$ form. CD4 is a monomer. In the CD8 receptor only the α -chain is associated with $p56^{lck}$.

15

Recent determinations of the physical parameters controlling binding of TCR and CD8 to MHC, using soluble versions of the receptors, have shown that binding by TCR dominates the recognition event. TCR has significantly higher affinity for MHC than the coreceptors.

20

The individual interactions of the receptors with MHC are very short-lived at physiological temperature, i.e. 37°C. An approximate figure for the half life of a TCR-MHC/peptide interaction, measured with a human TCR specific for the influenza virus "matrix" peptide presented by HLA-A*0201 (HLA-A2), is 0.7 seconds. The half life of the CD8 $\alpha\alpha$ interaction with the MHC/peptide complex is less than 0.01 seconds or at least 18 times faster (Willcox, *et al. Immunity* 10: 357-

25

65 (1999); Wyer, *et al. Immunity* 10: 219-225 (1999)).

Class I MHC restricted cellular immune responses are mediated by cytotoxic T cells (CTLs). CTLs are activated through protein-protein interactions between T cell
5 receptors (TCRs) and their coreceptors, CD8 molecules, on the T cell and MHC/peptide ligands on the target cell surface. TCRs form contacts both to the antigen peptide and to the heavy chain of the MHC complex (Ding, *et al. Immunity* 11: 45-56 (1999); Ding, *et al. Immunity* 8: 403-11 (1998); Garboczi, *et al. Nature* 384: 134-41 (1996); Garcia, *et al. Science* 274: 209-19 Issn: 0036-8075 (1996); and
10 provide the antigen specificity of the T cell. CD8 binds to the heavy chain and the light chain (β_2 -microglobulin) of the MHC complex, but not to the antigen peptide (Gao, *et al. Nature* 387: 630-4 (1997)). These protein binding events lead to activation of signal transduction in the T cell.

15 In contrast to antibody-antigen interactions, TCR-MHC/peptide interactions and, in particular, CD8-MHC interactions are characterised by low affinities and fast kinetics (Willcox, *et al. Immunity* 10: 357-65 (1999); Wyer, *et al. Immunity* 10: 219-225 (1999)). The interactions between T cells and antigen presenting cells are believed to be stabilised by multiple simultaneous receptor-ligand contacts,
20 increasing the avidity of cell-cell interactions (Mescher, *Immunol Rev* 146, 177-210 (1995); Norment, *et al. Nature* 336: 79-81 (1988)).

Cytotoxic T cells are exquisitely sensitive to interference that disrupts the normal kinetics and/or affinities of the interactions required for activation. For example,
25 altered peptide ligands (APLs) presented together with the normal antigen peptide on the MHC molecules of the antigen presenting cell can cause a failure to activate CTLs. Remarkably, this phenomenon can, in some cases, be observed even when the antagonist APL is presented at significantly lower molar ratios than the normal

antigen (Bertoletti, *et al. Nature* 369: 407-10 Issn: 0028-0836 (1994); Klenerman, *et al. Nature* 369: 403-7 (1994); Purbhoo, *et al. Proc. Natl. Acad. Sci. USA* 95: 4527-4532 (1998)). Thus, CTLs are highly sensitive to variations in TCR-antigen contacts, even if the majority of these are within the 'normal' parameters required for activation.

Suppressors of the cellular arm of the immune system, such as suppressors of CD4 or CD8 T cells, are urgently needed for the treatment of auto-immune disorders, such as rheumatoid arthritis, lupus erythematosus, psoriasis vulgaris, ankylosing spondylitis, Reiter's disease, post-salmonella arthritis, post-shigella arthritis, post-yersinia arthritis, post-gonococcal arthritis, uveitis, amyloidosis, idiopathic hemochromatosis and myasthenia gravis, and the prevention of graft rejection and graft-versus-host disease. Antibodies directed against CD4 and CD8 have been tried (De Fazio, *et al. Transplantation* 61: 104-10 (1996)), but with limited success and antibodies in general are not well suited as drugs since they tend to induce secondary immune responses and are short-lived. Administration of steroids is another way of suppressing the immune system but their effect is extremely indirect and associated with severe side-effects.

Attempts have been made to modulate the binding of CD8 to MHC class I molecules. One previous attempt involved the *in vitro* use of peptides derived from HLA sequences thought to interact with CD8 (Clayberger, *et al. J Immunol* 153: 946-51 (1994)). Two CD8 derived peptides that were also tested were found to be incapable of suppressing the differentiation of human CTL precursors into active effector cells and unable to inhibit the subsequent action of these effector cells.

Another attempt to modulate CTL responses using free CD8 derived peptides (Choksi, *et al. Nature Medicine* 4: 309-314 (1998)) showed that one peptide in

particular, "CSSHNKPC", could inhibit both the differentiation and effector stages of CTL response. However, a very high concentration of peptide ($> 100 \mu\text{g/ml}$) was required to bring about this inhibition ($> 50\%$).

- 5 CTL inhibition has also been observed with soluble CD8 $\alpha\alpha$ receptor (Sewell, *et al. Nature Medicine* 5: 399-404 (1999)). The inhibitory effect of the soluble CD8 molecule was more dramatic than that observed with an anti-CD8 monoclonal antibody.
- 10 In certain aspects, the present invention aims to prevent or inhibit CD8 $^{+}$ T cell responses by preventing or inhibiting CD8 binding to the β_2 -microglobulin subunit of the MHC/peptide complex.

According to a first aspect of the present invention, there is provided a method of
15 inhibiting the binding of a CD8 $^{+}$ T cell to a class I Major Histocompatibility Complex (MHC), the method comprising exposing the class I MHC to a modified β_2 -microglobulin whose binding to CD8 is inhibited.

In a second aspect, the invention provides a modified β_2 -microglobulin whose
20 binding to CD8 is inhibited for use in medicine.

In a third aspect the invention provides the use of a modified β_2 -microglobulin whose binding to CD8 is inhibited in the manufacture of a medicament for inhibiting CD8 $^{+}$ T cell response.

25

In a fourth aspect, the invention provides a method for the treatment of an autoimmune disorder, graft versus host disease or graft rejection, comprising

administering to a patient a modified β_2 -microglobulin whose binding to CD8 is inhibited.

As used herein "inhibited", when used in the context of the binding of one entity to another, means that the binding is prevented altogether or reduced to such a level that the normal physiological results of binding cannot be observed or are not significant. Even low levels of binding inhibition can have severe physiological effects (for example, low levels of soluble CD8 receptor can cause CTL inhibition (Sewell, *et al. Nature Medicine* 5: 399-404 (1999))). It would be expected that, *in vitro*, the binding of soluble CD8 molecule to a soluble HLA complex refolded with a modified β_2 -microglobulin may be inhibited by at least 20%, and more likely 50 to 100%.

In a preferred embodiment, the present invention relates to a means of inhibiting CD8⁺ T cell responses which is completely different to those proposed hitherto, in that CTLs are inhibited by preventing CD8 on the T cell surface from binding to MHC molecules on the antigen presenting cell by providing β_2 -microglobulin which has been modified such that its binding to CD8 is impaired or inhibited. This may be achieved by providing β_2 -microglobulin mutant proteins in which substitution, deletion and/or insertion mutations cause the binding of CD8 to be inhibited.

Substitution mutations are preferred as they create minimal disruption to the structure of β_2 m. The mutated residues, for instance sterically and/or electrostatically, prevent or impair the binding of CD8 to MHC complexes involving the mutant β_2 -microglobulin proteins. The observation that the inhibitory effect of soluble CD8 molecule is more dramatic than that observed with an anti-CD8 monoclonal antibody (Sewell, *et al. Nature Medicine* 5: 399-404 (1999)) indicates that CTL activity is more susceptible to inhibition by interference with CD8 binding at the target cell surface than by interference at the T cell surface.

- Because CD8 forms molecular contacts to both the MHC heavy chain and the light chain, β_2 -microglobulin, CD8 binding could, in principle, be inhibited by blocking the binding to either. However, the heavy chains of MHC complexes are membrane-bound and thus not well suited for the development of inhibitor proteins.
- 5 β_2 -microglobulin, on the other hand, is a soluble molecule that can be expressed as a recombinant protein (Gao, *et al. Prot. Sci.* 7: 1245-49 (1998); Garboczi, *et al. Proc Natl Acad Sci U S A* 89: 3429-33 Issn: 0027-8424 (1992); Parker & Wiley, *Gene* 83: 117-24 (1989)).
- 10 It is preferred if the modified β_2 -microglobulin is derived from human β_2 -microglobulin. The sequence for this protein is known (Swissprot P01884 EMBL/Genbank M17986). However, the modified β_2 -microglobulin may be derived from any species, e.g. dog, cat, rat or mouse. The sequences for rat and mouse β_2 -microglobulin are known (Rat: Swissprot P07151, EMBL/Genbank Y00441, Mouse:
- 15 Swissprot P01887 EMBL/Genbank X01838). For those β_2 -microglobulins which have not been crystallised and hence do not have data available as to which residues thereof are involved in binding CD8, the skilled person can identify likely candidate residues for mutation using homology studies.
- 20 Mutated versions of the β_2 m protein are known. However, none of these have been suggested for inhibiting class I MHC complex contacts to CD8. The most comprehensive set of mutations was investigated by Fukuzawa *et al* (Fukazawa, *et al. J Immunol* 153: 3543-50 (1994)) who generated 18 mutant β_2 m proteins. The aim of this study was to alter the conformation of HLA-B27 in order to change the
- 25 peptide presentation of the HLA complex. HLA-B27 is strongly associated with ankylosing spondylitis (Ivanyi, *Curr Opin Rheumatol* 4: 484-93 (1992); Reveille, *Am J Med Sci* 316: 239-49 (1998)) and is also associated with an increased frequency of certain autoimmune diseases (Altman, *et al. Ann Allergy* 72: 307-16 (1994);

Reveille, *Am J Med Sci* 316: 239-49 (1998)). The aim of Fukuzawa *et al* was to investigate whether T cell recognition of HLA-B27 could be modulated by β_2m mutant proteins, the idea being that the structure of the peptide binding groove of the HLA heavy chain may be affected by amino acid substitutions in β_2m (Fukazawa, *et al. J Immunol* 153: 3543-50 (1994)). The residues in the human β_2m that were mutated were positions 2, 4, 6, 10, 31, 32, 33, 51, 52, 53, 54, 55, 56, 59, 60, 63, 64, and 65. Some of these were only mutated in combination with other residues. Some mutations affected binding of a monoclonal antibody, Ye-2, to the HLA-B27 complex. The majority of the mutated proteins had reduced ability to exchange with β_2m on the cell surface, probably because the mutations affected residues involved in β_2m -heavy chain contacts (Fukazawa, *et al. J Immunol* 153: 3543-50 (1994)). The paper concludes that "It is unlikely that any of the mutants can be effective therapeutically in altering HLA-B27 function. Those mutants that are effective also exchange poorly into the complex." The authors only consider the possibility of modulating CTL activity through the influence β_2m mutations may have on peptide antigen presentation. The manuscript does not mention that mutations in β_2m might be used to modulate CTL activity by altering interactions with CD8. Furthermore, mutations at position 58 in β_2m are unlikely to have an adverse effect on the ability of the mutated β_2m polypeptide to exchange into HLA complexes as was observed with the majority of the mutant proteins analysed by Fukuzawa *et al*.

Another study has analysed the effects of single-residue mutations in the epitope of β_2m recognised by a panel of polyclonal antisera and by monoclonal antibodies (van Eyndhoven, *et al. Clin Immunol Immunopathol* 72: 362-72 (1994)). Mutations were concentrated in the region constituted by residues 57-63 of β_2m . The manuscript concludes 'that single residues within a small seven amino acid, solvent accessible antigenic stretch of primary beta $_2m$ sequence vary considerably in their contribution

to the reactive antigenic structures for IgM RF as well as monoclonal IgG antibodies.' The manuscript does not address the effect of the mutations on β_2m exchange, on HLA structure or on CD8 recognition.

- 5 A more recent study used murine β_2m mutants in exchange assays, followed by antibody recognition (Schultz, *et al.* *Immunogenetics* 48: 273-82 (1998)). This study did not address the issues of CTL reactivity or CD8 inhibition. Finally, a mutation of Tyrosine₆₇ to Cysteine in human β_2m has been described (Walter, *et al.* *J Immunol Methods* 214: 41-50 (1998)). The introduced Cysteine residue was used for
- 10 adding a biotin to the SH group, allowing soluble HLA complexes to be tetramerised through the linkage of biotin to avidin (Walter, *et al.* *J Immunol Methods* 214: 41-50 (1998)). Trymbulak *et al.* *Immunogenetics* 46: 418-426 (1997) also describes mutations to β_2m .
- 15 In accordance with a fifth aspect of the invention, there is provided a modified human β_2m in which Lysine₅₈ is replaced with one or more of Histidine, Tryptophan, Tyrosine, Phenylalanine, Glutamate, Aspartate, Glycine, Alanine, Valine, Leucine, Isoleucine, Methionine, Serine, Threonine, Asparagine, Glutamine, Proline and Cysteine. It is preferred if Lysine₅₈ is replaced with Arginine, Serine, Tyrosine,
- 20 Tryptophan, Cysteine, Serine-Glutamate-Serine, or Glycine-Arginine-Glycine, and especially preferred if replaced with Glutamate.

- In accordance with a sixth aspect of the invention, there is provided a modified human β_2m in which Serine₅₇ is replaced with one or more of Arginine, Histidine,
- 25 Tryptophan, Tyrosine, Phenylalanine, Glutamate, Aspartate, Glycine, Alanine, Valine, Leucine, Isoleucine, Methionine, Asparagine, Glutamine, Proline and Cysteine.

In accordance with a seventh aspect of the invention, there is provided a modified human β_2m in which Aspartate₅₉ is replaced with one or more of Arginine, Histidine, Tryptophan, Tyrosine, Phenylalanine, Glutamate, Glycine, Alanine, Valine, Leucine, Isoleucine, Methionine, Asparagine, Glutamine, Proline and Cysteine,
5 provided that, when mutated to Alanine, Phenylalanine₅₆ is not mutated to Tyrosine. It is preferred if Aspartate₅₉ is replaced with Glycine-Glutamate-Glycine.

In accordance with an eighth aspect of the invention, there is provided a modified human β_2m in which Tryptophan₆₀ is replaced with one or more of Arginine,
10 Histidine, Tyrosine, Phenylalanine, Glutamate, Aspartate, Glycine, Alanine, Valine, Leucine, Isoleucine, Methionine, Serine, Threonine, Asparagine, Glutamine, Proline and Cysteine, provided that:

- when mutated to Arginine, (i) Leucine₅₄ is not mutated to Valine and/or Leucine₆₄ is not mutated to Arginine;
- 15 when mutated to Cysteine, another residue is also mutated;
- when mutated to Leucine, Histidine₅₁ is not mutated to Tyrosine; and
- when mutated to Glycine, Aspartate₅₃ is not mutated to Tyrosine. It is preferred if Tryptophan₆₀ is replaced with Glycine.

20 In accordance with a ninth aspect of the invention, there is provided a modified human β_2m in which Serine₆₁ is replaced with one or more of Arginine, Histidine, Tryptophan, Tyrosine, Glutamate, Aspartate, Glycine, Alanine, Valine, Leucine, Isoleucine, Methionine, Threonine, Asparagine, Glutamine, Proline and Cysteine.

25 The invention also provides a modified human β_2m in which Lysine₅₈ is replaced with Arginine and Tryptophan₆₀ is replaced with Glycine.

In the fifth to ninth aspects of the invention, unless the context dictates otherwise, reference to "human β_2m " means a protein comprising amino acid sequence 1-99 in Figure 1a, and reference to numbered amino acid residues in human β_2m is in accordance with the numbering of the amino acid residues in Figure 1a. The invention also provides modified human β_2m with a combination of one or more of the mutations defined in the fifth to ninth aspects of the invention. The specific mutations mentioned may be the only mutations. Alternatively, there may be other mutations.

10 A modified human β_2m of the present invention may be provided in substantially pure form. For example, it may be provided in a form which is substantially free of other proteins.

The present invention encompasses any protein coded for by the nucleic acid sequences as shown in Figure 1a herein, when modified as shown in any one of Figures 1b-11.

The skilled person will appreciate that homologues or derivatives of the modified human β_2m proteins of the invention will also find use in the context of the present invention, i.e. in inhibiting the binding of a $CD8^+$ T cell to a class I Major Histocompatibility Complex (MHC). Thus, for instance proteins which include one or more additions, deletions, substitutions or the like are encompassed by the present invention. In addition, it may be possible to replace one amino acid with another of similar "type". For instance, replacing one hydrophobic amino acid with another. One can use a program such as the CLUSTAL program to compare amino acid sequences. This program compares amino acid sequences and finds the optimal alignment by inserting spaces in either sequence as appropriate. It is possible to calculate amino acid identity or similarity (identity plus conservation of amino acid type) for an optimal alignment. A program like BLASTx will align the longest stretch

of similar sequences and assign a value to the fit. It is thus possible to obtain a comparison where several regions of similarity are found, each having a different score. Both types of analysis are contemplated in the present invention.

- 5 In the case of homologues and derivatives, the degree of identity with a protein as described herein is less important than that the homologue or derivative should retain the ability to inhibit the binding of a CD8⁺ T cell to a class I Major Histocompatibility Complex. However, suitably, homologues or derivatives having at least 60% similarity (as discussed above) with the proteins or polypeptides described
10 herein are provided. Preferably, homologues or derivatives having at least 70% similarity, more preferably at least 80% similarity are provided. Most preferably, homologues or derivatives having at least 90% or even 95% similarity are provided.

- In an alternative approach, the homologues or derivatives could be fusion proteins,
15 incorporating moieties which render purification easier, for example by effectively tagging the desired protein or polypeptide. It may be necessary to remove the "tag" or it may be the case that the fusion protein itself retains sufficient ability to inhibit the binding of a CD8⁺ T cell to a class I Major Histocompatibility Complex to be useful.

- 20 The invention therefore provides, in a further aspect, a protein which is a homologue or derivative of the modified human β_2m proteins of the invention.

- The modified human β_2m proteins of the present invention can be provided alone, as a purified or isolated preparation. They may be provided as part of a mixture with
25 one or more other proteins of the invention.

In yet further aspects, the present invention provides:

- (a) the use of a modified human β_2m protein of the invention in inhibiting the binding of a $CD8^+$ T cell to a class I Major Histocompatibility Complex;
- (b) the use of a modified human β_2m protein of the invention in the production of a medicament for inhibiting $CD8^+$ T cell response; and
- (c) a method of inhibiting the binding of a $CD8^+$ T cell to a class I Major Histocompatibility Complex (MHC), the method comprising exposing the class I MHC to a modified human β_2m protein of the invention.

10

Gene cloning techniques may be used to provide a modified human β_2m protein of the invention in substantially pure form. These techniques are disclosed, for example, in J. Sambrook *et al Molecular Cloning* 2nd Edition, Cold Spring Harbor Laboratory Press (1989). Thus, in a further aspect, the present invention provides a nucleic acid

15 molecule comprising or consisting of a sequence which is:

- (i) the DNA sequence set out in Figure 1a herein, when modified as shown in any one of Figures 1b-11, or its RNA equivalent;
- (ii) a sequence which is complementary to the sequence of (i);
- (iii) a sequence which codes for the same protein or polypeptide, as the sequence of (i) or (ii);
- (iv) a sequence which has substantial identity with any of those of (i), (ii) and (iii);
- (v) a sequence which codes for a homologue, derivative or fragment of a protein as defined in Figure 1a, when modified as shown in any one of Figures 1b-11.

20

25

The nucleic acid molecules of the invention may include a plurality of such sequences, and/or fragments. The skilled person will appreciate that the present invention can

include novel variants of those particular novel nucleic acid molecules which are exemplified herein. Such variants are encompassed by the present invention. These may occur in nature, for example because of strain variation. For example, additions, substitutions and/or deletions are included. In addition, and particularly when utilising
5 microbial expression systems, one may wish to engineer the nucleic acid sequence by making use of known preferred codon usage in the particular organism being used for expression. Thus, synthetic or non-naturally occurring variants are also included within the scope of the invention.

10 The term "RNA equivalent" when used above indicates that a given RNA molecule has a sequence which is complementary to that of a given DNA molecule (allowing for the fact that in RNA "U" replaces "T" in the genetic code).

When comparing nucleic acid sequences for the purposes of determining the degree of
15 homology or identity, one can use programs such as BESTFIT and GAP (both from the Wisconsin Genetics Computer Group (GCG) software package). BESTFIT, for example, compares two sequences and produces an optimal alignment of the most similar segments. GAP enables sequences to be aligned along their whole length and finds the optimal alignment by inserting spaces in either sequence as appropriate.

20 Suitably, in the context of the present invention compare when discussing identity of nucleic acid sequences, the comparison is made by alignment of the sequences along their whole length.

Preferably, sequences which have substantial identity have at least 50% sequence
25 identity, desirably at least 75% sequence identity and more desirably at least 90 or at least 95% sequence identity with said sequences. In some cases, the sequence identity may be 99% or above.

Desirably, the term "substantial identity" indicates that said sequence has a greater degree of identity with any of the sequences described herein than with prior art nucleic acid sequences.

- 5 Substantially identical sequences may hybridise with the sequences of (i), (ii) and (iii) above under moderate or stringent hybridising conditions.

It should however be noted that, where a nucleic acid sequence of the present invention codes for at least part of a novel gene product, the present invention includes within its
10 scope all possible sequence coding for the gene product or for a novel part thereof.

The nucleic acid molecule may be in isolated or recombinant form. It may be incorporated into a vector and the vector may be incorporated into a host. Such vectors and suitable hosts form yet further aspects of the present invention.

15

As mentioned above, soluble β_2 -microglobulin protein, added to class I antigen presenting cells, is known to exchange with the protein that is part of the MHC complexes on the cell surface (Bernabeu, *et al. Nature* 308: 642-5 (1984); Cook, *et al. J Immunol* 157: 2256-61 (1996); Horig, *et al. Proc Natl Acad Sci U S A* 94: 13826-31 (1997); Hyafil & Strominger, *Proc Natl Acad Sci U S A* 76: 5834-8 (1979); Luscher, *et al. J Immunol* 153: 5068-81 (1994); Parker, *et al. J Immunol* 149: 1896-904 (1992); Smith, *et al. Proc Natl Acad Sci U S A* 89: 7767-71 (1992)). Experiments with a cell line have indicated that 10-25% of the exchange takes place within 10-15 minutes after addition of exogenous β_2 m (Luscher, *et al. J Immunol* 25 153: 5068-81 (1994)). Importantly, β_2 m exchange does not affect the stability of peptide binding, so antigen presentation is not lost or altered on the MHC molecules which undergo β_2 m exchange (Cook, *et al. J Immunol* 157: 2256-61 (1996); Horig, *et al. Proc Natl Acad Sci U S A* 94: 13826-31 (1997); Parker, *et al. J Immunol* 149:

1896-904 (1992); Smith, *et al. Proc Natl Acad Sci U S A* 89: 7767-71 (1992)).

Thus, recombinant β_2 -microglobulin proteins, modified or mutated so as to prevent CD8 forming the normal binding complex with the MHC (heavy chain/light chain/peptide) molecule, can be added to antigen presenting cells to act as an
5 immune inhibitor. The modified β_2 m can be delivered to the antigen presenting cell surface either by exchange or by expression of a mutated β_2 m gene in the cells, as is explained in more detail below.

Binding of the CD8 $\alpha\alpha$ homodimer to the class I HLA complex is asymmetric, the
10 two subunits of CD8 being involved in different contacts. Only one of the CD8 subunits is involved in binding β_2 m, through its β strand 'A' in the immunoglobulin structure. The contributions of the β_2 m-CD8 α contacts to binding have not been established. However, considering the low affinity of CD8 $\alpha\alpha$ binding to HLA complex (Wyer, *et al. Immunity* 10: 219-225 (1999)), it is likely that disruption of
15 any contacts will lead to a significant inhibition of coreceptor's contribution to signal transduction in T cells.

Two residues in human β_2 -microglobulin are the preferred targets for mutation. These are Lysine₅₈ and Tryptophan₆₀, both of which are involved in contacts with
20 CD8 (Gao, *et al. Nature* 387: 630-4 (1997)). Lysine₅₈ is the most preferred target. CD8 fits tightly into the binding 'cavity' formed by the heavy chain/light chain complex (Gao, *et al. Nature* 387: 630-4 (1997)). In the CD8 $\alpha\alpha$ -HLA-A2 crystal structure, Lysine₅₈ protrudes from the β_2 m loop towards the cavity occupied by CD8 (Gao, *et al. Nature* 387: 630-4 (1997)) and forms contacts to the CD8 residues
25 Arginine₄, Valine₂₄, Leucine₂₅, Leucine₂₆ and Aspartate₇₅. Therefore, amino acids with larger side chains, for instance arginine or tyrosine, at position 58 of β_2 -microglobulin have severe effects on CD8 binding. Aspartic acid or glutamic acid residues may also be used for substituting Lysine₅₈, since these exercise electrostatic

repulsion of CD8. Even fairly inconspicuous residues inserted in place of Lysine₅₈, for example serine, abrogate CD8 binding due to loss of specificity. In addition, CD8 binding may be inhibited by deleting Lysine₅₈ or inserting one or more residues which disrupt the cavity occupied by CD8.

5

It is known that mutations in the β_2m polypeptide can severely impair its ability to exchange into the HLA complex. One residue, mutation of which was found to impair the ability of the protein to exchange, was Tryptophan₆₀ (Fukazawa, *et al.*: *J Immunol* 153: 3543-50 (1994)). However, Lysine₅₈ is located in a loop, not a strand, of β_2m and is not involved in contacts with the class I heavy chain.

10

Therefore amino acid substitutions of Lysine₅₈ should not impair the ability of the protein to exchange into the HLA complex. Lysine₅₈ is therefore the prime candidate residue for substitutions designed to generate a soluble protein, that can exchange with β_2m on the cell surface and serve to inhibit CTL activation by impairing CD8 contacts to the MHC complex.

15

The preferred mutations of Lysine₅₈ and the rationale for how they might change CD8 recognition, and inhibit this, are as follows:

20

Lysine₅₈ → Arginine (R). Arginine has a larger, and different, side chain than lysine, and therefore this substitution is likely to sterically hinder CD8 from slotting correctly into the cavity formed by the HLA complex.

Lysine₅₈ → Glutamate (E). The side chain of glutamic acid is oppositely charged to lysine, and therefore this substitution is likely to electrostatically repel CD8 binding to HLA complexes.

25

Lysine₅₈ → Serine (S). The side chain of serine lacks the positive charge of the lysine side chain. The lack of the electrostatic interactions to CD8 in

which Lysine₅₈ is involved is probably sufficient to weaken CD8 binding to HLA complexes.

5 Lysine₅₈ → Tyrosine (Y). Tyrosine has a substantially larger, and under normal conditions uncharged, side chain than lysine. This substitution is likely to sterically hinder CD8 binding to HLA complexes.

Lysine₅₈ → Cysteine (C). Mutation to cysteine creates a 'free' SH group on the surface of β_2m being contacted by CD8. This may in itself inhibit CD8 contacts, but the residue could also be used to derivatise the β_2m polypeptide.

10 Figure 1a of the accompanying drawings shows the amino acid sequence of the processed human β_2m polypeptide and the DNA sequence encoding it, and Figures 1b-1 show the modifications that need to be made to this DNA sequence to introduce the mutations described above, as well as other mutations, and the amino acid sequences of the relevant regions of resulting β_2m proteins.

15

Tryptophan₆₀ is another preferred residue for mutation as it is involved in forming a contact to Arginine₄ in CD8 α (Gao *et al.*, *Nature* 387:630-4 (1997)). However, data published in Fukazawa, *et al. J Immunol* 153: 3543-50 (1994) suggests that mutation of this residue may interfere with the ability of the mutant protein to exchange.

20 Thus, it is preferred that such mutant proteins are exposed to antigen presenting cells by means other than exchange, such as by expression of the mutant protein in the cell.

In addition to Lysine₅₈ and Tryptophan₆₀, because of the low affinity and transient
25 nature of CD8-MHC interactions, functionally significant effects may be achieved by quite a large number of mutations (whether they be insertion, deletion or substitution mutations) in residues 57-61 of human β_2m (i.e. Serine₅₇, Aspartate₅₉ and Serine₆₁) either singly or in combination.

Substitution mutations of these residues can be divided into categories as follows:

- Mutation to Arginine, Histidine, Tryptophan, Tyrosine or Phenylalanine. These residues have large side chains and are therefore likely to cause steric obstruction of CD8 binding if introduced into the loop contacted by the coreceptor.
5
- Mutation to Glutamate or Aspartate. These residues have negatively charged side chains and are likely to cause electrostatic repulsion of CD8 binding, since Aspartate₇₅ of human CD8 α is involved in contacts to β_2m (Gao, *et al. Nature* 387: 630-4 (1997)).
- 10 • Mutation to Glycine, Alanine, Valine, Leucine, Isoleucine, Methionine, Serine, Threonine, Asparagine or Glutamine. Some of these residues have small side chains, and all are uncharged at normal physiological pH. Mutations of one or several of residues 57-61 of human β_2m to any of this set of residues could cause loss of CD8 binding, either sterically or simply because the normal contacts
15 between CD8 and β_2m are lost.
- Mutation to Proline. Proline induces structural restrictions in polypeptides and would therefore be predicted to alter the structure of the 57-61 loop of human β_2m , with expected loss of CD8 binding to the MHC complex. However, introduction of a Proline residue(s) may not be compatible with a functional β_2m
20 structure, that is, folding of β_2m may be affected in such a way that the protein cannot form complexes with HLA heavy chains or have impaired ability to exchange into existing complexes on surface of cells.
- Mutation to Cysteine. Mutation to cysteine would create a 'free' SH group on the surface of β_2m being contacted by CD8. This may in itself inhibit CD8
25 contacts, but the residue could also be used to derivatise the β_2m polypeptide. For instance, a biotin group could be linked to β_2m , causing steric hindrance of

CD8 binding if the mutated, derivatised β_2m polypeptide was exchanged into MHC complexes.

In addition to the mutations described above, β_2m may be mutated to increase its
5 affinity for MHC so that its on-rate is greater and its off-rate is less than wild type β_2m , thereby resulting in greater occupancy of the mutant β_2m in the MHC.

Mutations of this type are described in Schultz, *et al. Immunogenetics* 48: 273-82 (1998).

10 Other possible mutations will be apparent to those skilled in the art.

Mutant β_2m proteins of the present invention can be expressed as soluble recombinant protein for extracellular addition, or expressed intracellularly by transfection of a DNA construct encoding the mutant β_2m protein. Transfection of
15 DNA can be achieved both *in vitro* as well as *in vivo*, for example by using various type of recombinant viruses as vehicles for DNA transformation or by transfection techniques that use 'naked' DNA. For example, an organ to be transplanted may be incubated in a modified β_2m to make it more difficult for the immune system of host to recognise and reject it.

20

Preferably in gene therapy, the modified β_2m protein is administered such that it is expressed in the subject to be treated for example in the form of a recombinant DNA molecule comprising a polynucleotide encoding the modified β_2m protein operatively linked to a nucleic acid sequence which controls expression, such as in an expression
25 vector. Such a vector will thus include appropriate transcriptional control signals including a promoter region capable of expressing the coding sequence, said promoter being operable in the subject to be treated. Thus for human gene therapy, the promoter, which term includes not only the sequence necessary to direct RNA

- polymerase to the transcriptional start site, but also, if appropriate, other operating or controlling sequences including enhancers, is preferably a human promoter sequence from a human gene, or from a gene which is typically expressed in humans, such as the promoter from human cytomegalovirus (CMV). Among known eukaryotic
- 5 promoters suitable in this regard are the CMV immediate early promoter, the HSV thymidine kinase promoter, the early and late SV40 promoters, the promoters of retroviral LTRs, such as those of the Rous sarcoma virus ("RSV"), and metallothionein promoters, such as the mouse metallothionein-I promoter.
- 10 A polynucleotide sequence and transcriptional control sequence may be provided cloned into a replicable plasmid vector, based on commercially available plasmids, such as pBR322, or may be constructed from available plasmids by routine application of well known, published procedures.
- 15 The vector may also include transcriptional control signals, situated 3' to the mutant β_2m encoding sequence, and also polyadenylation signals, recognisable in the subject to be treated, such as, for example, the corresponding sequences from viruses such as, for human treatment, the SV40 virus. Other transcriptional controlling sequences are well known in the art and may be used.
- 20 The expression vectors may also include selectable markers, such as for antibiotic resistance, which enable the vectors to be propagated.
- 25 Expression vectors capable *in situ* of synthesising mutant β_2m may be introduced directly by physical methods. Examples of these include topical application of the 'naked' nucleic acid vector in an appropriate vehicle for example in solution in a pharmaceutically acceptable excipient such as phosphate buffered saline (PBS). Other physical methods of administering the DNA directly to the recipient include ultrasound, electrical stimulation, electroporation and microseeding.

Mutant β_2m encoding nucleic acid sequence for use in the therapy of the invention may also be administered by means of delivery vectors. These include viral delivery vectors, such as adenovirus or retrovirus delivery vectors known in the art. Other non-
5 viral delivery vectors include lipid delivery vectors, including liposome delivery vehicles, known in the art.

A mutant β_2m encoding nucleic acid sequence may also be administered by means of transformed host cells. Such cells include cells harvested from the subject, into which
10 the nucleic acid sequence is introduced by gene transfer methods known in the art, followed by growth of the transformed cells in culture and administration to the subject.

Expression constructs such as those described above may be used in a variety of ways
15 in the therapy of the present invention. Thus, they may be directly administered to the subject, or they may be used to prepare mutant β_2m itself which can then be administered as is discussed in more detail below. The invention also relates to host cells which are genetically engineered with constructs which comprise mutant β_2m encoding polynucleotide, and to the uses of these vectors and cells in the therapeutic
20 methods of the invention. These constructs may be used *per se* in the therapeutic methods of the invention or they may be used to prepare a mutant β_2m polypeptide for use in the therapeutic methods of the invention described in greater detail below.

The vector may be, for example, a plasmid vector, a single or double-stranded phage
25 vector, a single or double-stranded RNA or DNA viral vector, depending upon whether the vector is to be administered directly (i.e. for *in situ* synthesis), or is to be used for synthesis of mutant β_2m . Starting plasmids disclosed herein are either commercially available, publicly available, or can be constructed from available plasmids by routine application of well known, published procedures. Many plasmids

and other cloning and expression vectors that can be used in accordance with the present invention are well known and readily available to those of skill in the art.

- Generally, vectors for expressing a mutant β_2m polypeptide for use in the invention
- 5 comprise cis-acting control regions effective for expression in a host operatively linked to the polynucleotide to be expressed. Appropriate trans-acting factors either are supplied by the host, supplied by a complementing vector or supplied by the vector itself upon introduction into the host.
- 10 In certain embodiments in this regard, the vectors provide for specific expression. For production of mutant β_2m , such specific expression may be inducible expression or expression only in certain types of cells or both inducible and cell-specific. Particularly preferred among inducible vectors are vectors that can be induced for expression by environmental factors that are easy to manipulate, such as temperature
- 15 and nutrient additives. A variety of vectors suitable to this aspect of the invention, including constitutive and inducible expression vectors for use in prokaryotic and eukaryotic hosts, are well known and employed routinely by those of skill in the art.
- A great variety of expression vectors can be used to express mutant β_2m for use in the
- 20 invention. Such vectors include, among others, chromosomal, episomal and virus-derived vectors, e.g., vectors derived from bacterial plasmids, from bacteriophage, from transposons, from yeast episomes, from insertion elements, from yeast chromosomal elements, from viruses such as baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and
- 25 retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids, all may be used for expression in accordance with this aspect of the present invention. Generally, any vector suitable to maintain, propagate or express polynucleotides to express a polypeptide in a host may be used for expression in this regard.

The appropriate DNA sequence may be inserted into the vector by any of a variety of well-known and routine techniques, such as, for example, those set forth in Sambrook et al., MOLECULAR CLONING, A LABORATORY MANUAL, 2nd Ed.; Cold
5 Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989).

The nucleic acid sequence in the expression vector may be operatively linked to appropriate expression control sequence(s), including, for instance, a promoter to direct mRNA transcription. Representatives of such promoters include, but are not
10 limited to, the phage lambda PL promoter, the *E. coli* lac, trp and tac promoters, for recombinant expression, and the SV40 early and late promoters and promoters of retroviral LTRs for *in situ* expression.

In general, expression constructs will contain sites for transcription initiation and
15 termination, and, in the transcribed region, a ribosome binding site for translation. The coding portion of the mature transcripts expressed by the constructs will include a translation initiating AUG at the beginning and a termination codon appropriately positioned at the end of the polypeptide to be translated.

20 In addition, the constructs may contain control regions that regulate as well as engender expression. Generally, in accordance with many commonly-practised procedures, such regions will operate by controlling transcription, such as transcription factors, repressor binding sites and termination, among others.

25 Vectors for propagation and expression generally will include selectable markers and amplification regions, such as, for example, those set forth in Sambrook et al., MOLECULAR CLONING, A LABORATORY MANUAL, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989).

Representative examples of appropriate hosts for recombinant expression of mutant β_2m include bacterial cells, such as *streptococci*, *staphylococci*, *E. coli*, *streptomyces* and *Bacillus subtilis* cells; fungal cells, such as yeast cells and *Aspergillus* cells; insect cells such as *Drosophila* S2 and *Spodoptera* Sf9 cells; animal or human cells such as
5 CHO, COS, HeLa, C127, 3T3, BHK, 293 and Bowes melanoma cells; and plant cells.

The following vectors, which are commercially available, are provided by way of example. Among vectors preferred for use in bacteria are pQE70, pQE60 and pQE-9, available from Qiagen; pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A,
10 pNH16a, pNH18A, pNH46A, available from Stratagene; and ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia, and pBR322 (ATCC 37017). Among preferred eukaryotic vectors are pWLNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; and pSVK3, pBPV, pMSG and pSVL available from Pharmacia. These vectors which can be used both for recombinant expression and for
15 *in situ* expression are listed solely by way of illustration of the many commercially available and well known vectors that are available to those of skill in the art for use in accordance with this aspect of the present invention. It will be appreciated that any other plasmid or vector suitable for, for example, introduction, maintenance, propagation or expression of a polynucleotide or polypeptide for use in the therapy of
20 the invention in a host may be used in this aspect of the invention.

Examples of vectors for use in this aspect of the invention include expression vectors in which the mutant β_2m cDNA sequence is inserted in a plasmid whereby gene expression is driven from the human immediate early cytomegalovirus enhancer-
25 promoter (Foecking and Hofstetter, *Cell*, 45, 101-105, 1986). Such expression plasmids may contain SV40 RNA processing signals such as polyadenylation and termination signals. Expression constructs which use the CMV promoter and that are commercially available are pCDM8, pCDNA1 and derivatives, pCDNA3 and derivatives (Invitrogen). Other expression vectors available which may be used are

pSVK3 and pSVL which contain the SV40 promoter and mRNA splice site and polyadenylation signals from SV40 (pSVK3) and SV40 VP1 processing signals (pSVL; vectors from Pharmacia).

- 5 Promoter regions can be selected from any desired gene using vectors that contain a reporter transcription unit lacking a promoter region, such as a chloramphenicol acetyl transferase ("CAT") transcription unit, downstream of restriction site or sites for introducing a candidate promoter fragment; i.e., a fragment that may contain a promoter. As is well known, introduction into the vector of a promoter-containing
- 10 fragment at the restriction site upstream of the cat gene engenders production of CAT activity, which can be detected by standard CAT assays. Vectors suitable to this end are well known and readily available, such as pKK232-8 and pCM7. Promoters for expression of polynucleotides for use in the therapy of the present invention include not only well known and readily available promoters, but also promoters that readily
- 15 may be obtained by the foregoing technique, using a reporter gene; for *in situ* expression, such a promoter should be recognised in the subject to be treated.

Among known prokaryotic promoters suitable for expression of polynucleotides and polypeptides in accordance with the therapy of the present invention are the *E. coli*

20 lacI and lacZ and promoters, the T3 and T7 promoters, the gpt promoter, the lambda PR, PL promoters and the trp promoter.

Recombinant expression vectors will include, for example, origins of replication, a promoter preferably derived from a highly-expressed gene to direct transcription of a

25 downstream structural sequence, and a selectable marker to permit isolation of vector containing cells after exposure to the vector.

Polynucleotides for use in the therapy of the invention, encoding the mutant or modified β_2m polypeptide generally will be inserted into the vector using standard

techniques so that it is operably linked to the promoter for expression. The polynucleotide will be positioned so that the transcription start site is located appropriately 5' to a ribosome binding site. The ribosome binding site will be 5' to the AUG that initiates translation of the polypeptide to be expressed.

5

Generally, there will be no other open reading frames that begin with an initiation codon, usually AUG, and lie between the ribosome binding site and the initiation codon. Also, generally, there will be a translation stop codon at the end of the polypeptide and there will be a polyadenylation signal in constructs for use in
10 eukaryotic hosts. Transcription termination signal appropriately disposed at the 3' end of the transcribed region may also be included in the polynucleotide construct.

For secretion of the translated protein into the lumen of the endoplasmic reticulum, into the periplasmic space or into the extracellular environment, appropriate secretion
15 signals may be incorporated into the expressed polypeptide when recombinantly synthesised. These signals may be endogenous to the polypeptide or they may be heterologous signals.

The polypeptide may be expressed in a modified form, such as a fusion protein, and
20 may include not only secretion signals but also additional heterologous functional regions. Thus, for instance, a region of additional amino acids, particularly charged amino acids, may be added to the N- or C-terminus of the polypeptide to improve stability and persistence in the host cell, during purification or during subsequent handling and storage. Also, a region may be added to the polypeptide to facilitate
25 purification. Such regions may be removed prior to final preparation of the polypeptide. The addition of peptide moieties to polypeptides to engender secretion or excretion, to improve stability or to facilitate purification, among others, are familiar and routine techniques in the art. A preferred fusion protein comprises a heterologous region from immunoglobulin that is useful to solubilise or purify polypeptides. Cells

typically then are harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification.

Microbial cells employed in expression of proteins can be disrupted by any convenient
5 method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents, such methods are well known to those skilled in the art.

Mammalian expression vectors may comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites,
10 polyadenylation regions, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking non-transcribed sequences that are necessary for expression.

For preparing mutant β_2m polypeptides for use in the invention, genetically engineered
15 host cells may be used. Introduction of a polynucleotide into the host cell can be affected by calcium phosphate transfection, DEAE-dextran mediated transfection, transvection, microinjection, cationic lipid-mediated transfection, electroporation, transduction, scrape loading, ballistic introduction, infection or other methods. Such methods are described in many standard laboratory manuals, such as Davis et al.,
20 BASIC METHODS IN MOLECULAR BIOLOGY, (1986) and Sambrook et al., MOLECULAR CLONING: A LABORATORY MANUAL, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y (1989).

Mature proteins can be expressed in host cells including mammalian cells such as
25 CHO cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by

Sambrook et al., MOLECULAR CLONING: A LABORATORY MANUAL, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989).

The polypeptide can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulphate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography is employed for purification. Well known techniques for refolding protein may be employed to regenerate active conformation when the polypeptide is denatured during isolation and or purification.

Assays for β_2m exchange both on purified MHC/peptide complexes and on the cell surface (Hyafil & Strominger, *Proc Natl Acad Sci U S A* 76: 5834-8 (1979); Luscher, *et al. J Immunol* 153: 5068-81 (1994)) can be employed to investigate the ability of mutant β_2m polypeptides to exchange into MHC/peptide complexes. However, more convenient assays, with which an initial assessment of β_2m exchange and inhibition of CD8 binding can be obtained, are envisaged. Binding of CD8 to MHC/peptide complexes can be conveniently detected by surface plasmon resonance studies, for instance on the Biacore2000 or Biacore3000 systems (Garcia, *et al. Nature* 384: 577-81 Issn: 0028-0836 (1996); Wyer, *et al. Immunity* 10: 219-225 (1999)). Thus, CD8 binding to sensor cells with immobilised MHC/peptide complexes could be measured, one cell serving as control for other sensor cells on which the MHC/peptide complex has been exposed to β_2m exchange with mutant proteins. Such analysis will provide a biophysical measurement of the degree to which the mutant β_2m proteins are capable of exchanging and inhibiting CD8 binding. The production of soluble MHC-peptide complexes is well known. Soluble MHC-peptide complexes were first obtained by cleaving the molecules of the surface

of antigen presenting cells with papain (Bjorkman, *et al. J Mol Biol* **186**: 205-10 (1985)). Although this approach provided material for crystallisation, it has, for class I molecules, in recent years been replaced by individual expression of heavy and light chain in *E.coli* followed by refolding in the presence of synthetic peptide
5 (Gao, *et al. Prot. Sci.* **7**: 1245-49 (1998); Gao, *et al. Nature* **387**: 630-4 (1997); Garboczi, *et al. Proc Natl Acad Sci U S A* **89**: 3429-33 Issn: 0027-8424 (1992); Garboczi, *et al. J Mol Biol* **239**: 581-7 Issn: 0022-2836 (1994); Madden, *et al.* [published erratum appears in *Cell* 1994 Jan 28;76(2):following 410]. *Cell* **75**: 693-708 Issn: 0092-8674 (1993); Reid, *et al. J Exp Med* **184**: 2279-86 (1996); Reid, *et*
10 *al. FEBS Lett* **383**: 119-23 (1996); Smith, *et al. Immunity* **4**: 215-28 Issn: 1074-7613 (1996); Smith, *et al. Immunity* **4**: 203-13 Issn: 1074-7613 (1996)). This approach has several advantages over previous methods in that a better yield is obtained at a lower cost, peptide identity can be controlled very accurately, and the final product is more homogenous. Furthermore, expression of modified heavy or light chain, for
15 instance fused to a protein tag, can be easily performed.

The inhibitory effects of mutant β_2m proteins can be tested in *in vitro* CTL assays in order to assess their inhibitory effect on T cell activation. These studies can be extended to *in vivo* analysis of the effects of the mutant β_2m proteins, by testing
20 these in relevant animal disease models. The outcome of such studies can form the basis on which further pre-clinical studies of the immune-inhibitory effects of the mutated β_2m polypeptides are carried out.

Medicaments in accordance with the invention will usually be supplied as part of a
25 sterile, pharmaceutical composition which will normally include a pharmaceutically acceptable carrier. This pharmaceutical composition may be in any suitable form, (depending upon the desired method of administering it to a patient).

It may be provided in unit dosage form, will generally be provided in a sealed container and may be provided as part of a kit. Such a kit would normally (although not necessarily) include instructions for use. It may include a plurality of said unit dosage forms.

5

The pharmaceutical composition may be adapted for administration by any appropriate route, for example by the oral (including buccal or sublingual), rectal, nasal, topical (including buccal, sublingual or transdermal), vaginal or parenteral (including subcutaneous, intramuscular, intravenous or intradermal) route. Such compositions may
10 be prepared by any method known in the art of pharmacy, for example by admixing the active ingredient with the carrier(s) or excipient(s) under sterile conditions.

Pharmaceutical compositions adapted for oral administration may be presented as discrete units such as capsules or tablets; as powders or granules; as solutions, syrups
15 or suspensions (in aqueous or non-aqueous liquids; or as edible foams or whips; or as emulsions)

Suitable excipients for tablets or hard gelatine capsules include lactose, maize starch or derivatives thereof, stearic acid or salts thereof. Suitable excipients for use with soft
20 gelatine capsules include for example vegetable oils, waxes, fats, semi-solid, or liquid polyols etc.

For the preparation of solutions and syrups, excipients which may be used include for example water, polyols and sugars. For the preparation of suspensions oils (e.g.
25 vegetable oils) may be used to provide oil-in-water or water in oil suspensions.

Pharmaceutical compositions adapted for transdermal administration may be presented as discrete patches intended to remain in intimate contact with the epidermis of the recipient for a prolonged period of time. For example, the active ingredient may be

delivered from the patch by iontophoresis as generally described in *Pharmaceutical Research*, 3(6):318 (1986).

Pharmaceutical compositions adapted for topical administration may be formulated as
5 ointments, creams, suspensions, lotions, powders, solutions, pastes, gels, sprays,
aerosols or oils. For infections of the eye or other external tissues, for example mouth
and skin, the compositions are preferably applied as a topical ointment or cream.
When formulated in an ointment, the active ingredient may be employed with either a
paraffinic or a water-miscible ointment base. Alternatively, the active ingredient may
10 be formulated in a cream with an oil-in-water cream base or a water-in-oil base.
Pharmaceutical compositions adapted for topical administration to the eye include eye
drops wherein the active ingredient is dissolved or suspended in a suitable carrier,
especially an aqueous solvent. Pharmaceutical compositions adapted for topical
administration in the mouth include lozenges, pastilles and mouth washes.

15

Pharmaceutical compositions adapted for rectal administration may be presented as
suppositories or enemas.

Pharmaceutical compositions adapted for nasal administration wherein the carrier is a
20 solid include a coarse powder having a particle size for example in the range 20 to 500
microns which is administered in the manner in which snuff is taken, i.e. by rapid
inhalation through the nasal passage from a container of the powder held close up to the
nose. Suitable compositions wherein the carrier is a liquid, for administration as a
nasal spray or as nasal drops, include aqueous or oil solutions of the active ingredient.

25

Pharmaceutical compositions adapted for administration by inhalation include fine
particle dusts or mists which may be generated by means of various types of metered
dose pressurised aerosols, nebulizers or insufflators.

Pharmaceutical compositions adapted for vaginal administration may be presented as pessaries, tampons, creams, gels, pastes, foams or spray formulations.

- 5 Pharmaceutical compositions adapted for parenteral administration include aqueous and non-aqueous sterile injection solution which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation substantially isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. Excipients which may be used
10 for injectable solutions include water, alcohols, polyols, glycerine and vegetable oils, for example. The compositions may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection
15 solutions and suspensions may be prepared from sterile powders, granules and tablets.

- The pharmaceutical compositions may contain preserving agents, solubilising agents, stabilising agents, wetting agents, emulsifiers, sweeteners, colourants, odourants, salts (substances of the present invention may themselves be provided in the form of a
20 pharmaceutically acceptable salt), buffers, coating agents or antioxidants. They may also contain therapeutically active agents in addition to the substance of the present invention.

- Dosages of the substances of the present invention can vary between wide limits,
25 depending upon the disease or disorder to be treated, the age and condition of the individual to be treated, etc. and a physician will ultimately determine appropriate dosages to be used. The dosage may be repeated as often as appropriate. If side

effects develop the amount and/or frequency of the dosage can be reduced, in accordance with normal clinical practice.

Preferred features of each aspect of the invention are as for each of the other aspects
5 *mutatis mutandis*. The prior art documents mentioned herein are incorporated to the fullest extent permitted by law.

The invention will now be described further in the following non-limiting examples. Reference is made to the accompanying drawings in which:

10

Figure 1a shows the amino acid sequence of the processed human β_2m polypeptide and the DNA sequence encoding it, and Figures 1b-1 show the modifications that need to be made to this DNA sequence to introduce mutations in accordance with the invention, and the amino acid sequences of the relevant regions of resulting β_2m

15

proteins;

Figure 2 shows a Coomassie-stained SDS-PAGE gel of the *Escherichia coli* strain BL21-DE3 (pLysS) expressing human β_2m ;

20

Figure 3 shows a Coomassie-stained SDS-PAGE gel with purified inclusion bodies containing denatured human β_2m ;

Figure 4 shows a gel filtration trace of refolded human β_2m , the correctly folded protein eluting after 220 ml;

25

Figure 5 shows a SDS-PAGE gel with size markers in Lane 1 and refolded β_2m in Lane 2;

Figure 6 shows schematically a procedure for determining the ability of β_2m mutant proteins to exchange and to inhibit cytotoxic lymphocyte activation;

Figures 7a-e show BIAcore responses showing the ability of FLU-HLA-A2/ β_2m complexes containing mutated β_2m to bind soluble CD8 $\alpha\alpha$ compared to that of complexes containing wild type β_2m ;

5

Figures 8a-e show BIAcore responses showing the ability of HLA-A2/ β_2m complexes containing mutated β_2m to bind TCR compared to that of complexes containing wild type β_2m ;

10 Figures 9a and 9b show BIAcore responses showing the ability of HLA-A2/ β_2m complexes containing mutated β_2m to bind CD8 and TCR respectively after storage for 24 hours, compared to that of complexes containing wild type β_2m ;

Figure 10 is a plasmid map of the plasmid pIRES;

15

Figure 11 shows the coding sequence of HLA-A*02011, together with primer sequences used in the present invention;

Figure 12 shows the coding sequence for full length wild type β_2m ;

20

Figure 13 is a graph illustrating the inhibition of HLA-A2 restricted CTL clone 5D8 by mutant HLA/ β_2m complexes;

Figure 14 is a graph illustrating the inhibition of HLA-A2 restricted parvovirus B19 specific CTL line by mutant HLA/ β_2m complexes; and

25

Figure 15 shows schematically a procedure for investigating human β_2m inhibition of CTL priming *in vivo*.

Examples

Example 1 – K₅₈ → E mutation of human β_2m

- 5 This example describes the construction of the DNA expression plasmid pEX052 which codes for the β_2m in which Lysine₅₈ is substituted for Glutamate. The DNA sequences of the primers used are shown highlighted in Figure 1b (K₅₈ → E); the sequence on top corresponds to the sense strand of β_2m , the sequence on the bottom corresponds to the non-sense strand of β_2m . Mutated bases are indicated in small
10 letters and the amino acid sequence is indicated below the DNA sequences.

- A DNA plasmid, pBJ192, which encodes human β_2m in which the signal peptide is substituted for a single Methionine residue in order to allow initiation of translation when expressed in bacteria, was generated as follows. A PCR reaction was performed
15 on cDNA generated from a human B cell line with the primers 5'- GGG GGG CAT ATG ATt CAa aGa ACT CCA Aaa ATT CAG GTT TAC TCA CGT CAT CC -3' (forward primer) and 5'- GGG GGA AGC TTA CAT GTC TCG ATC CCA CTT AAC TAT – 3' (backward primer). Bases shown in small letters indicate mutations in relation to the human sequence. These do not alter the sequence of the polypeptide
20 that is encoded, in relation to the human sequence, but were introduced in order to increase expression of the β_2m protein in *E. coli*. The PCR product was cloned into pGMT7 (Studier, *et al. Methods Enzymol* 185: 60-89 Issn: 0076-6879 (1990)) at the restriction sites for NdeI and HindIII.

- 25 100 ng of plasmid pBJ192 was mixed with 5 μ l 10 mM dNTP, 25 μ l 10xPfu-buffer (Stratagene), 10 units Pfu polymerase (Stratagene) and the final volume was adjusted to 240 μ l with H₂O. 48 μ l of this mix was supplemented with primers diluted to give a final concentration of 0.2 μ M in 50 μ l final reaction volume. After an initial denaturation step of 30 seconds at 95° the reaction mixture was subjected to 15 rounds

of denaturation (95°C, 30 sec.), annealing (55°C, 60 sec.), and elongation (73°C, 8 min.) in a Hybaid PCR express PCR machine. The product was then digested for 5 hours at 37°C with 10 units of DpnI restriction enzyme (New England Biolabs). 10 µl of the digested reaction was transformed into XL1-Blue bacteria and grown for 18 hours at 37°C. A single colony was picked and grown over night in 5 ml TYP + ampicillin (16 g/l Bacto-Tryptone, 16 g/l Yeast Extract, 5 g/l NaCl, 2.5 g/l K₂HPO₄, 100 mg/l Ampicillin). Plasmid DNA was purified on a Qiagen mini-prep column according to the manufacturer's instructions and the sequence was verified by automated sequencing at the sequencing facility of Department of Biochemistry, Oxford University.

Example 2 – K₅₈ → S mutation of human β₂m

This example describes the construction of the DNA expression plasmid pEX053 which codes for the β₂m in which Lysine₅₈ is substituted for Serine. The DNA sequences of the primers used are shown in Figure 1c (K58 → S). Mutation of DNA plasmid pBJ192 was carried out according to the same protocol as described in Example 1 and the sequence was verified by automated sequencing at the sequencing facility of Department of Biochemistry, Oxford University.

Example 3 – K₅₈ → R mutation of human β₂m

This example describes the construction of the DNA expression plasmid pEX051 which codes for the β₂m in which Lysine₅₈ is substituted for Arginine. The DNA sequences of the primers used are shown in Figure 1d (K58 → R). Mutation of DNA plasmid pEX052 was carried out according to the same protocol as described in Example 1 except that 20 PCR rounds were carried out and the elongation time was increased to 10 minutes. The sequence was verified by automated sequencing at the sequencing facility of Department of Biochemistry, Oxford University.

Example 4 – K₅₈ → Y mutation of human β_2m

This example describes the construction of the DNA expression plasmid pEX054
5 which codes for the β_2m in which Lysine₅₈ is substituted for Tyrosine. The DNA
sequences of the primers used are shown in Figure 1e (K58 → Y). Mutation of DNA
plasmid pEX052 was carried out according to the same protocol as described in
Example 3 and the sequence was verified by automated sequencing at the sequencing
facility of Department of Biochemistry, Oxford University.

10

Example 5 – K₅₈ → C mutation of human β_2m

This example describes the construction of the DNA expression plasmid pEX055
which codes for the β_2m in which Lysine₅₈ is substituted for Cysteine. The DNA
15 sequences of the primers used are shown in Figure 1f (K58 → C). Mutation of DNA
plasmid pEX052 was carried out according to the same protocol as described in
Example 3 and the sequence is verified by automated sequencing at the sequencing
facility of Department of Biochemistry, Oxford University.

20 *Example 6 – K₅₈ → SES mutation of human β_2m*

This example describes the construction of the DNA expression plasmid pEX056
which codes for the β_2m in which Lysine₅₈ is substituted for Serine-Glutamate-Serine.
The DNA sequences of the primers used are shown in Figure 1g (K58 → SES).
25 Mutation of DNA plasmid pEX052 was carried out according to the same protocol as
described in Example 3 and the sequence was verified by automated sequencing at the
sequencing facility of Department of Biochemistry, Oxford University.

Example 7 – K₅₈ → W mutation of human β_2m

This example describes the construction of the DNA expression plasmid pEX061 which codes for the β_2m in which Lysine₅₈ is substituted for Tryptophan. The DNA sequences of the primers used are shown in Figure 1h (K58 → W). Mutation of DNA
5 plasmid pEX051 was carried out according to the following protocol.

10 ng of template plasmid (pEX051) was mixed with 1.25 μ l 10 mM dNTP, 5 μ l 10xPfu-buffer (Stratagene), 2.5 units Pfu polymerase (Stratagene) and the final
10 volume was adjusted to 50 μ l with H₂O. 48 μ l of this mix was supplemented with primers diluted to give a final concentration of 0.2 μ M in 50 μ l final reaction volume. After an initial denaturation step of 2 minutes at 95°C the reaction mixture was subjected to 18 rounds of denaturation (95°C, 30 sec.), annealing (55°C, 60 sec.), and elongation (68°C, 10 min.) in a Hybaid PCR express PCR machine. The product was
15 then digested for 1 hour at 37°C with 20 units of DpnI restriction enzyme (New England Biolabs). 10 μ l of the digested reaction was transformed into XL1-Blue bacteria and grown for 18 hours at 37°C. A single colony was picked and grown over night in 5 ml TYP + ampicillin (16 g/l Bacto-Tryptone, 16 g/l Yeast Extract, 5 g/l NaCl, 2.5 g/l K₂HPO₄, 100 mg/l Ampicillin). Plasmid DNA was purified on a Qiagen
20 Spin mini-prep column according to the manufacturer's instructions and the sequence was verified by automated sequencing at the sequencing facility of Department of Biochemistry, Oxford University.

Example 8 – K₅₈ → GRG mutation of human β_2m

25

This example describes the construction of the DNA expression plasmid pEX062 which codes for the β_2m in which Lysine₅₈ is substituted for the tri-peptide Glycine-Arginine-Glycine. The DNA sequences of the primers used are shown in Figure 1i (K58 → GRG). Mutation of DNA plasmid pEX056 was carried out according to the

same protocol as described in Example 7 and the sequence was verified by automated sequencing at the sequencing facility of Department of Biochemistry, Oxford University.

5 *Example 9 – D₅₉ → GEG mutation of human β_2m*

This example describes the construction of the DNA expression plasmid pEX063 which codes for the β_2m in which Aspartate₅₉ is substituted for the tri-peptide Glycine-Glutamate-Glycine. The DNA sequences of the primers used are shown in Figure 1j
10 (D59 → GEG). Mutation of DNA plasmid pEX050 was carried out according to the same protocol as described in Example 7 and the sequence was verified by automated sequencing at the sequencing facility of Department of Biochemistry, Oxford University.

15 *Example 10 – W₆₀ → G mutation of human β_2m*

This example describes the construction of the DNA expression plasmid pEX064 which codes for the β_2m in which Tryptophan₆₀ is substituted for Glycine. The DNA sequences of the primers used are shown in Figure 1k (W60 → Y). Mutation of DNA
20 plasmid pEX050 was carried out according to the same protocol as described in Example 7 and the sequence was verified by automated sequencing at the sequencing facility of Department of Biochemistry, Oxford University.

Example 11 – K₅₈W₆₀ → RG mutation of human β_2m

25

This example describes the construction of the DNA expression plasmid pEX065 which codes for the β_2m in which Lysine₅₈ and Tryptophan₆₀ are substituted for Arginine and Glycine respectively. The DNA sequences of the primers used are shown in Figure 1l (K58W60 → RG). Mutation of DNA plasmid pEX051 was
30 carried out according to the same protocol as described in Example 7 and the sequence

was verified by automated sequencing at the sequencing facility of Department of Biochemistry, Oxford University.

Example 12 - Expression, refolding and purification of human β_2m and β_2m mutants

5

β_2m protein was expressed from the DNA vector pBJ192, and from mutated derivatives of pBJ192, e.g. pEX051-56 and pEX061-65, in the *Escherichia coli* strain BL21-DE3 pLysS (Novagen). pBJ192 contains the β_2m gene under the control of the strongly inducible T7 promoter in the vector pGMT7 (Studier, *et al. Methods Enzymol* 10 185: 60-89 Issn: 0076-6879 (1990)). The BL21 cells transformed with one of the β_2m -expressing vectors were plated on LB/agar/100 mg/ml ampicillin plates made according to a standard recipe. Transformants were then grown in TYP medium with Ampicillin (16 g/l Bacto-Tryptone, 16 g/l Yeast Extract, 5 g/l NaCl, 2.5 g/l K_2HPO_4 , 100 mg/l Ampicillin) to an $OD_{600} \sim 0.4$. For large-scale expression, 1 l volumes of 15 TYP media were prepared in 2 l conical flasks and were covered with four layers of aluminium foil and were autoclaved. Cell densities were measured using optical density at 600 nm wavelength (OD_{600}) on a Beckman DU530 spectrophotometer. Sterile TYP media was used as a blank. Figure 2 shows a Coomassie-stained SDS-PAGE gel of the *Escherichia coli* strain BL21-DE3 (pLysS) expressing human β_2m . 20 Lane 1 contains a bacterial extract before induction of T7 transcription, lane 2 shows an extract from the same bacterial culture, but after 3 hours of induction of T7 transcription. Prior to induction, very little β_2m was produced by the bacteria, but after induction with 0.5 mM Iso-phenyl thio-galactoside (IPTG, from Melford), large amounts of β_2m were produced and deposited in inclusion bodies. Inclusion bodies 25 were purified as described (Gac, *et al, Prot. Sci.* 7: 1245-49 (1998)). Cells were lysed in 'Lysis Buffer' (10 mM EDTA (from 0.5 M stock pH 8.0), 2 mM DTT (from 1 M stock in 10 mM sodium acetate pH 5.2, stored at $-20^\circ C$), 10 mM Tris pH 8.1 (from 2 M stock pH 8.1), 150 mM NaCl (from 4 M stock), 200 $\mu g/ml$ lysozyme (from 20 mg/ml stock stored at $-20^\circ C$), 10% glycerol (from fluid), 2500 units of DNAase I and

10mM MgCl₂ using a 50 ml Dounce homogeniser DNase I and lysozyme were from Sigma). Sonication, in lysis buffer, to break open the cells was performed using a 12mM probe sonicator (Misonix XL2020). The probe was tuned according to the manufacturers instructions. The resulting suspension was then diluted 1:1 in 'Triton Buffer' (0.5% (w/v) Triton X-100 (from fluid), 50 mM Tris pH 8.1 (from 2 M stock), 100 mM NaCl (from 4 M stock), 0.1% sodium azide (from solid), 10 mM EDTA (from 0.5 M stock pH 8.0), 2 mM DTT (from 1 M stock in 10 mM sodium acetate pH 5.2, stored at -20°C) and left overnight. The inclusion bodies were separated from cell debris by centrifugation in a Beckman J2-21 centrifuge equipped with a JA-20 rotor as described (Gao, *et al*, *Prot. Sci.* 7: 1245-49 (1998)) and stored at -20°C. Inclusion bodies were then thawed and resuspended in 'Resuspension Buffer' (50 mM Tris pH 8.1 (from 2 M stock), 100 mM NaCl (from 4 M stock), 10 mM EDTA (from 0.5 M stock pH 8.0), 2 mM DTT (from 1 M stock in 10 mM sodium acetate pH 5.2, stored at -20°C)), and denatured in 6M Guanidine and 10mM DTT buffered with Tris-HCl pH 8.1 (all chemicals from Sigma). Figure 3 shows a Coomassie-stained SDS-PAGE gel with purified inclusion bodies containing denatured human β_2m . β_2m is then refolded *in vitro* in the presence of 0.4M L-Arginine and purified by gel filtration chromatography, for instance on a Pharmacia Superdex 75 column. Figure 4 shows a gel filtration trace of refolded human β_2m , the correctly folded protein eluting after 220 ml. β_2m protein typically expresses at a level >100 mg/l medium and refolds at an efficiency of ~36% giving high yields of correctly refolded soluble protein. It elutes from the Superdex 75 PG (preparation grade 26/60) column (Pharmacia) at an elution volume of 220 ml. It was judged, by Coomassie-stained SDS-PAGE, to be >95% pure after the single purification step. Figure 5 shows a SDS-PAGE gel with size markers in Lane 1 and refolded β_2m in Lane 2.

Example 13 - Testing of CD8 binding to HLA complex incorporating β_2m mutant proteins

β_2m mutant proteins were prepared according to Example 12, using the plasmids described in Examples 1-11. After the purification of denatured β_2m mutant protein, refolding was carried out in the presence of peptide and denatured HLA heavy chain (Gao, *et al*, *Prot. Sci.* 7: 1245-49 (1998)) containing a tag sequence that can be enzymatically biotinylated (Schatz, *Biotechnology N Y* 11: 1138-43 (1993); Altman, *et al Science* 274: 94-6 (1996); Wyer, *et al. Immunity* 10: 219-225 (1999)). The complex was then biotinylated using the enzyme BirA (O'Callaghan, *et al. Anal Biochem* 266(1): 9-15 (1999)) to produce mutant β_2m - HLA-A2 - peptide complexes which were biotinylated towards the C-terminus of the HLA-A2 heavy chain. These protein complexes were immobilised on a streptavidin-modified BIAcore chip sensor cell in a BIAcore 3000 machine. The binding of soluble CD8 $\alpha\alpha$ were monitored by surface plasmon resonance when the CD8 protein was caused to flow over the sensor cell at a concentration in the range of 10-20 mg/ml.

Determination of the effects on CD8 binding of mutations introduced in the β_2m protein was accomplished with an experimental set up as illustrated in Figure 6. The HLA (or MHC) complex involving non-mutated β_2m protein was immobilised in one sensor cell, the HLA complexes involving each mutated β_2m protein was immobilised in each other sensor cell. To test that all complexes were correctly folded, soluble TCR, specific for the HLA/peptide complex used, was passed through all the sensor cells used (Wyer, *et al. Immunity* 10: 219-225 (1999)). Detection of the antigen specific interaction is an extremely strong indication that folding of the HLA complex is correct. Next, soluble CD8 protein was passed over all of the sensor cells and the levels of binding in these were compared. If binding of soluble CD8 in a sensor cell was absent, or significantly reduced, then it was concluded that the mutant β_2m protein incorporated into this complex had affected CD8's ability to bind the complex.

The following β_2m mutants were prepared.

	<u>Mutation</u>	<u>Plasmid</u>
5	58K \rightarrow R	pEX 051
	58K \rightarrow E	pEX 052
	58K \rightarrow S	pEX 053
	58K \rightarrow Y	pEX 054
	58K \rightarrow C	pEX 055
10	58K \rightarrow SES	pEX 056
	58K \rightarrow W	pEX061
	58K \rightarrow GRG	pEX062
	59D \rightarrow GEG	pEX063
	60W \rightarrow G	pEX064
15	59K/60W \rightarrow R/G	pEX065

The ability of HLA-A2/ β_2m complexes containing the above mutations to bind soluble CD8 $\alpha\alpha$ was compared to that of complexes containing WT β_2m , as described above.

- 20 The results of studies to determine the binding of HLA-A2/mutant β_2m complexes to bind soluble CD8 $\alpha\alpha$ are shown in Figures 7a-e.

The concentration in μM is calculated from the measured absorbance of the sCD8 $\alpha\alpha$ stock solution ($\text{Conc} = \text{Abs} \times 10^6 / (\text{extinct co} \times \text{Mw})$). The response is the difference
 25 between the response obtained for one cell containing a complex and the reference cell and is indicated in Response Units. The concentration of sCD8 $\alpha\alpha$ in μM was plotted (x) against the response in RU (y) and the points were fitted in the equation:
 $\text{Response} = n[\text{CD8}] / ([\text{CD8}] + K_d)$.

The ability of the HLA/mutant β_2m complexes to be conveniently refolded and to bind to the appropriate TCR, were also investigated. The results are shown in Figures 8a-e. The concentration of TCR μM was plotted (x) against the response in RU (y) and the points were fitted in the equation $Response = n[TCR]/([TCR] + K_d)$.

The stability of the HLA-A2-mutant β_2m complexes was also investigated for the 58K \rightarrow E and 59D \rightarrow GEG mutants by re-assessing their ability to bind CD8 and TCR after storage at room temperature on a BIA core chip for 24 hours. The results are shown in Figures 9a and 9b respectively. The 24 hour stability assessment demonstrated no change in the CD8 or TCR binding response for the 58K \rightarrow E mutant. However the TCR binding of the 59D \rightarrow GEG mutant demonstrated a drop in total binding response with a practically unchanged K_d .

15

The results obtained (see Figures 7a-e) demonstrate that the HLA-A2 β_2m substitution mutations cause a decrease in affinity for soluble CD8 $\alpha\alpha$. Almost total inhibition of CD8 binding was caused by the following mutations:

20 K58 \rightarrow SES, K58 \rightarrow E, K58 \rightarrow Y, W60 \rightarrow G, K58/W60 \rightarrow R/G, K58 \rightarrow W, D59 \rightarrow GEG and K58 \rightarrow GRG.

Partial inhibition of CD8 binding was caused by the following mutations, in order of decreasing inhibition:

25

K58 \rightarrow R > K58 \rightarrow C > K58 \rightarrow S.

The recovery on refolding of the mutants was also assessed (see Figures 8a-e) and the mutants are listed in order of decreasing recovery:

K58→R = K58→E > K58→Y = K58→W > K58→C = K58→S > K58→SES
 5 = D59→GEG = K58→GRG > W60→G = K58/W60→R/G

The ability of the mutants to bind TCR was also assessed (see Figures 8a-e) and the mutants tested are listed in order of decreasing total binding response:

10 K58→R = D59→GEG = K58→E > K58→Y = K58→W > K58→SES =
 K58→GRG = W60→G = K58/W60→R/G (K58→C & K58→S not tested)

Among the mutants that totally inhibit CD8 binding, only K58→E refolds as well as WT β_2m and is fully functional. The 24 hour storage tests indicate that the K58→E
 15 mutant is stable over this time period whereas the D59→GEG mutant undergoes partial degradation.

Example 14 – Compounds which may be attached to Cysteine in the β_2m K₅₈ → C mutant and linkers which may effect the attachment.

20

Mutation of Lysine₅₈ in β_2m to a Cysteine residue allows the unpaired SH group of the Cysteine to be used to link other compounds to the mutated β_2m protein. Such other compounds are used sterically to hinder CD8 binding to HLA (or MHC) complexes of which the mutated and derivatised β_2m forms a part. This strategy is
 25 similar to those described above where mutation to residues with larger side chains are used to prevent or inhibit CD8 binding. The difference with using derivatisation of the SH group of Cysteine at position 58 is that relatively larger compounds can be incorporated into the protein. This can cause a more efficient steric hindrance than

can be achieved with non-derivatised amino acid mutations. However, this involves a further step in the preparation and may reduce the ability of the protein to exchange into HLA/peptide complexes.

- 5 Compounds that can be used to derivatise β_2m K₅₈ → C include amino acids, peptides, Biotin, and DNA, all of which would be expected to severely affect CD8's ability to slot into its binding site on HLA (or MHC) complexes.

- Derivatisation is performed with a suitable linker molecule, such as Maleimide,
10 Iodoacetamide, 3, -(pyridyldithio)propionate and 3, -(pyridyldithio)propionamide, all of which are commercially available from chemical reagent companies (Sigma, Aldrich, Pierce). These linker compounds can be used to attach an almost limitless variety of groups to a Cysteine residue, either by producing a linker-derivatised molecule (e.g. 3-(N-Maleimidopropionyl) Biocytin™ available from Sigma) or by
15 using a heterobifunctional molecule such as e-Maleimidocaproic acid N-hydroxysuccinimide ester (Sigma) to conjugate e.g. a protein.

Example 15 – HLA/mutant β_2m mammalian expression vector for immuno-suppressive therapy

20

- There are a number of expression systems, known to those skilled in the art, which can be appropriate for the therapeutic delivery of the proteins of the present invention. DNA can be delivered by a number of different established routes using viral vectors, injection of naked DNA into the recipient tissue in a formulation
25 optimised for cellular uptake, injection using accelerated DNA coated particles, or any technology designed for *in vivo* gene delivery.

HLA/mutant $\beta 2m$ complexes can be administered against CTL based immune disorders using modified adeno-associated virus. This is a vector which can be used for the therapeutic delivery of DNA, and is capable of infecting a wide range of tissue types and therefore could be used for a variety of applications.

5

Tissue specificity of expression can be controlled by using tissue specific promoter and enhancer sequences in conjunction with a general mode of delivery. Tissue specificity can also be obtained by using viral vectors which only infect certain cell or tissue types in conjunction with using general regulatory elements controlling the expression of the genes. The vector and regulatory sequence combination of choice will depend on the nature of the disease being treated.

The human creatine kinase (CKM) promoter is an example of a tissue-specific regulatory sequence capable of targeted DNA expression in muscle tissue. The human cytomegalovirus (CMV) promoter is an example of a regulatory sequence capable of inducing DNA expression in a broad range of tissues.

15

Example 16 – Production of HLA- $\beta 2m$ mammalian expression vector for in vitro T cell assays

20

This example details the protocol used to produce an appropriate bicistronic vector for the production of wild type (WT) and HLA-mutant $\beta 2m$ expression vectors, which can be used to induce expression of these proteins for *in-vitro* T cell assays.

25 10^7 T2 cells (Payne *et al*, Immunogenetics (1990) 31(3):169-78) cells were harvested and lysed in 1 ml of TRI reagent from Sigma and total RNA was harvested according to instructions provided by the manufacturer. 10 μ g total RNA, 0.01 OD₂₆₀ of d(pT)₁₂₋₁₈ primer, 10 μ l of 10xRT buffer and 5 μ l Omniscript Reverse

Transcriptase (Qiagen Omniscript RT-kit Cat. No. 205111 lot EGQ002) was used for cDNA synthesis in a total volume of 100 µl according to instructions provided by the manufacturer. PCR was performed on 0.5 µl T2 cDNA in a total volume 40 µl using the primers A2-F1 (aaacccgggtctagaggatggccgcatggcg cc) and A2-R1 (cccgcgccgctcacactttacaagctgtgagagac) at 0.5 µM each, 0.2 mM dNTP, and 2 units of cloned Pfu DNA polymerase (Stratagene # 600153). The programme used an initial 10 minutes denaturation step at 95°C followed by 30 cycles of denaturation for 1 minute at 95°C, annealing for 1 min at 50°C, and elongation for 4 minutes at 73°C and a final elongation step at 73°C. The product was purified after electrophoresis on a 1% agarose gel by electro-transfer to GF/C filter, elution from the filter by centrifugation, extraction by Phenol:Chloroform:Isoamyl alcohol (25:24:1) and chromatography purified on a Superdex G50 Spincolumn. The purified PCR product and 200 ng pIRES (Clontech #6028-1) was digested with 10 units XbaI and 5 units Not I in a final volume of 20 µl Tris-Acetate buffer (33 mM Tris Acetate pH 7.9, 66 KOAc, 10mM MgOAc, 0.1 mg/ml autoclaved gelatin, 0.5 mM DTT). Digests were purified from an 0.9% agarose/TBE gel using the same technique as described above. The fragment was ligated into pIRES (see Figure 10) using a Rapid DNA Ligation Kit (Roche # 1 635 379) according to the manufacturers instructions. The entire HLA-A*0201 coding sequence (see Figure 11) was cloned between the XmaI or the XbaI site and the NotI site in MCS B. The design of the oligo-nucleotides allows amplification of this sequence from human cDNA. The insert sequence of the resulting plasmid, pEX060, was verified by automated sequencing.

The gene coding for full length wild type β2m (see Figure 12) was amplified from total T cell RNA by RT-PCR using primers β2m-F2 (cccagctagctcgagatgtctcgctccgtggct) and β2m-R3 (aaacacgcgttacatgtctcgatcccactta) and cloned XhoI-MluI into pIRES by standard techniques as described above. The

insert sequence of the resulting plasmid, pEX066, was verified by automated sequencing.

Different genes coding for full length mutant β 2m proteins were prepared by PCR-stitching using primers β 2m-F2 + β 2m-R2 (tccacttttcaattctctctccatt) on T cell cDNA template for the 5'-end and primers β 2m-F3 (cctgaattgctatgtgtctgggtt) and β 2m-R3 on plasmid pEX051, pEX052, or pEX56 as template for the 3'-end followed by PCR amplification of the mixed products using β 2m-F2 and β 2m-R3 and cloned Xho I - Mlu I into pIRES by standard techniques as described above. The insert sequences of the resulting plasmids, pEX067 (K58→E), pEX068 (K58→SES), and pEX069 (K58→R) were verified by automated sequencing.

The HLA-A*02011 fragment from pEX060 was subcloned by standard techniques Xba I - Not I into each of plasmids pEX066, pEX067, pEX068, and pEX069 to give bi-cistronic expression plasmids pEX070 (wt. + HLA-A*02011), pEX071(K58→E + HLA-A*02011), pEX072(K58→SES + HLA-A*02011), and pEX073(K58→R + HLA-A*02011) respectively.

The vector can be used to induce transient expression of HLA-A*0201 combined with wild type- or mutant β 2m in appropriate human target cells. The transfected target cell can be any HLA-A*0201 negative Antigen presenting cell (APC), for example, C1R, T293 or HeLa cells. Transfected and peptide labelled cells can be used as targets for lysis by HLA-A*0201 specific CTLs in a standard chromium release assay, or similar.

Example 17 – Assay for Immune-inhibitory effect of HLA-mutant β 2m complexes

The ability of target cells transfected with HLA-mutant β 2m complexes as described in Example 16 to inhibit *in vitro* CTL activity is determined by the following methodology.

A human T cell line (enriched and selected), recognising the target HLA e.g. HLA-A*0201 is used. A constant number of target cells (5000), expressing the HLA-A*0201- β 2m complex, are used and the number of T cells are varied. 'E:T' represents a ratio of numbers of T cells to target cells. Four E:T ratios are tested. The peptide concentrations are varied from 10^{-11} to 10^{-7} M. Two sets of experiments probing the mutant HLA-A*0201-mutant β 2m complex effect are conducted as follows:

- Group 1 - incubation with target cells expressing only the WT HLA-A*0201- β 2m complex as a control;
- Group 2 - incubation with target cells expressing HLA-A2*0201-mutant - β 2m complex.

The assay components for these experiments are: 18 μ l 10X peptide; 18 μ l PBS; 50 μ l containing 5000 target cells; 100 μ l containing 5000 –50000 CTL. Results are collected after 2 hours incubation.

The inhibition of CTL activity in cells expressing the HLA A2*0201-mutant - β 2m complexes indicates their immune-suppressive activity.

Example 18 – Inhibition of HLA-A2 restricted CTL clone 5D8 by HLA/ mutant β 2m complexes

Target cells (T2 hybrids) were grown in RPMI containing 10% human serum for 5
5 days. These cells were then incubated with wild-type or mutant β 2m protein
(K58→R, K58→Y, K58→E, K58→SES) at 300 μ g/ml in RPMI medium containing
1 μ M peptide (SLYNTVATL, GAG P17-HIV1) for 2 hours. These cells were then
washed and labelled with ^{51}Cr for 1 hour in the presence of 150 μ g/ml wild-type or
mutant β 2m protein. After further washes, the target cells were plated out with CTL
10 at a range of E:T ratios. Wild-type or mutant β 2m protein was present at 300 μ g/ml
for the duration of the assay. Supernatants were harvested after 2 hours.

The results are shown in Figure 13, in which it can be seen that all of the mutants
tested exhibited an inhibition of CTL activity at E:T ratios of 1:1 or greater.

15

*Example 19 – Inhibition of HLA-A2 restricted parvovirus B19 specific CTL line by
HLA/mutant β 2m complexes*

Target cells (T2 hybrids) were grown in RPMI containing 10% bovine serum for 5
20 days. These cells were then washed and labelled with ^{51}Cr for 1 hour. The target
cells were then incubated with either wild-type β 2m or mutant β 2m (K58→E) at
30 μ g/ml in RPMI medium containing 100 nM peptide (epitope is EADVQQWLTW)
for 15 minutes. The target cells were plated out with CTL (CTL line) at a ratio of 22:1
(E:T ratio). Wild-type (WT) β 2m or mutant β 2m was present at 30 μ g/ml for the
25 duration of the assay. Supernatants were harvested after 4 hours.

The results are shown in Figure 14. The addition of mutant β 2m protein resulted in a
specific lysis of 2.6% compared to 7.3% in the presence of WT β 2m protein. This
demonstrates the ability of mutant β 2m protein to inhibit CTL activity.

Example 20 – Test for Immunogenicity of mutant β -2-microglobulin

- Mutant β -2-microglobulin molecules could potentially induce an immune response either by antibodies and/or T cells. The introduction of mutation(s) in the β -2-microglobulin protein could potentially introduce a conformational change in the protein structure, which would be recognised by antibodies as a structural foreign antigen or, alternatively, enzymatic degradation of mutant β -2-microglobulin could produce peptides not normally presented by self MHC molecules. These mutant peptides would then be recognised as foreign and induce a cellular immune response. Therefore, mutants can be tested in a transgenic rat model expressing human MHC class I molecule (HLA-B27 heavy chain + β -2-microglobulin) as follows.
1. Inject transgenic rats with 3-4mg mutant beta-2-microglobulin.
 2. Collect serum from rats after 21 days.
 3. Analyse serum for the production of anti-mutant β -2-microglobulin antibodies by an ELISA. The procedure for this ELISA is: a. Bind mutant beta-2-microglobulin to bottom of well; b. Add serum from transgenic rat, which has been treated with mutant beta-2-microglobulin; c. wash three times with 200 μ L of wash buffer; d.add the appropriate concentration of conjugated anti-rat antibody; e. wash three times with 200 μ L of wash buffer; f. add 100 μ L detection reagent (Alkaline Phosphatase, substrate pNPP) and read absorbance at 405nm. Absorbance readings above negative control readings will indicate, that anti-mutant β -2-microglobulin antibodies are present in the serum.

Example 21 - Inhibition of CTL priming and activity by mutant HLA/ β 2m complexes in vivo.

The ability of mutant β 2m to inhibit a cellular immune response *in vivo* is tested by
5 three experiments in mouse, assaying the influence of mutant β 2m on the response
to an epitope encoded in a vaccinia virus (See Figure 15).

Materials and reagents:

Mice: In all three experiments, mice of strain Black 6 (C57BL/6 wildtype
10 laboratory mouse strain) are used.

Virus: Vaccinia virus strain G2 (vvG2) is a recombinant virus strain. Infection of
cells with Vaccinia virus generally results in the MHC restricted presentation of a
number of vaccinia peptide epitopes which are poorly characterised. Infection with
strain vvG2, however, also results in the presentation of a peptide epitope,
15 corresponding to amino acids 33-41 (sequence in one-letter code: KAVYNFATC)
from the LCM virus G2 glycoprotein. In mice, the G2 epitope is presented by the
MHC molecule D^b.

Target cells : MC57 antigen presenting cells (Leist, et al (1987) J Immunol 138:
2278-81) express mouse MHC molecules K^b and D^b.

20

Experiment 1:

A control group of mice are injected, intravenously, at Day 0 with 2×10^6 pfu (plaque
forming units) vaccinia virus strain G2 plus 200 μ l phosphate buffered saline (PBS,
standard physiological saline buffer). A test group of mice are also injected,
25 intravenously, at Day 0 with 2×10^6 pfu Vaccinia virus strain G2 plus 200 μ l mutant
 β 2m protein (20mg/ml) in PBS buffer.

To assay CTL activity, a modification of a previously described procedure can be used (Leist, *et al* (1987) *J Immunol* 138: 2278-81). On Day 6, the spleens are removed from the mice and lymphocytes washed out. Spleen cells are cultured in 24-well plates. Synthetic peptide corresponding to the G2 33-41 epitope is added to the wells and lymphocytes allowed to proliferate for four days. On Day 10, T cells are counted and CTL killing assays are performed as follows. Chromium⁵¹-labelled MC57 cells are prepulsed with G2 33-41 peptide for one hour, then mixed with the cultured mouse lymphocytes at a range of effector to target ratios. Chromium⁵¹ counts from wells of MC57 cells lysed by addition of detergent are set at 100% lysis.

Experiment 2:

The control and test group of mice are treated identically to those in Experiment 1 except that injections are performed intraperitoneally. CTL activity is assessed as described in Experiment 1.

Experiment 3:

A control group of mice are injected intraperitoneally with 200 μ l phosphate buffered saline. This dose is administered five hours prior to infection vaccinia virus strain G2, and a further injection 24 hours after the first one. A test group of mice is injected intraperitoneally with mutant β 2m protein (4 mg) in PBS buffer. This dose is administered five hours prior to infection vaccinia virus strain G2, and a further injection 24 hours after the first one.

CTL activity is assessed as described in Experiment 1.

Claims

1. A method of inhibiting the binding of a CD8⁺ T cell to a class I Major Histocompatibility Complex (MHC), the method comprising exposing the class I
5 MHC to a modified β_2 -microglobulin whose binding to CD8 is inhibited.
2. A method as claimed in claim 1, wherein the modified β_2 -microglobulin differs from wild type β_2 -microglobulin by virtue of one or more deletion, substitution and/or insertion mutations.
- 10 3. A method as claimed in claim 2, wherein the modified β_2 -microglobulin differs from wild type β_2 -microglobulin by virtue of one or more mutated residues which sterically and/or electrostatically inhibit or impair the binding of CD8 to MHC complexes including the mutant β_2 -microglobulin.
- 15 4. A method as claimed in claim 1, 2 or 3, wherein the modified β_2 -microglobulin is derived from human β_2 -microglobulin.
5. A method as claimed in claim 4, wherein the mutated residue is one or more
20 of Serine₅₇, Lysine₅₈, Aspartate₅₉, Tryptophan₆₀ and Serine₆₁.
6. A method as claimed in claim 5, wherein the mutated residue is mutated to Cysteine which is derivatised.
- 25 7. A method as claimed in claim 5 or claim 6, wherein Lysine₅₈ is replaced with Arginine, Glutamate, Serine, Tyrosine, Tryptophan, Cysteine, Serine-Glutamate-Serine, or Glycine-Arginine-Glycine.

8. A method as claimed in claim 7, wherein Lysine₅₈ is replaced with Glutamate.
9. A method as claimed in claim 5, wherein Aspartate₅₉ is replaced with
5 Glycine-Glutamate-Glycine.
10. A method as claimed in claim 5, wherein Tryptophan₆₀ is replaced with Glycine.
- 10 11. A method as claimed in claim 5, wherein Lysine₅₈ is replaced with Arginine, and Tryptophan₆₀ is replaced with Glycine.
12. A modified human β_2m in which Lysine₅₈ is replaced with one or more of Histidine, Tryptophan, Tyrosine, Phenylalanine, Glutamate, Aspartate, Glycine,
15 Alanine, Valine, Leucine, Isoleucine, Methionine, Serine, Threonine, Asparagine, Glutamine, Proline and Cysteine.
13. A modified human β_2m as claimed in claim 12, wherein Lysine₅₈ is replaced with Arginine, Glutamate, Serine, Tyrosine, Tryptophan, Cysteine, Serine-
20 Glutamate-Serine, or Glycine-Arginine-Glycine.
14. A modified human β_2m in which Serine₅₇ is replaced with one or more of Arginine, Histidine, Tryptophan, Tyrosine, Phenylalanine, Glutamate, Aspartate, Glycine, Alanine, Valine, Leucine, Isoleucine, Methionine, Asparagine, Glutamine,
25 Proline and Cysteine.
15. A modified human β_2m in which Aspartate₅₉ is replaced with one or more of Arginine, Histidine, Tryptophan, Tyrosine, Phenylalanine, Glutamate, Glycine,

Alanine, Valine, Leucine, Isoleucine, Methionine, Asparagine, Glutamine, Proline and Cysteine, provided that, when mutated to Alanine, Phenylalanine₃₆ is not mutated to Tyrosine.

5 16. A modified human β_2m as claimed in claim 15, wherein Aspartate₅₉ is replaced with Glycine-Glutamate-Glycine.

17. A modified human β_2m in which Tryptophan₆₀ is replaced with one or more of Arginine, Histidine, Tyrosine, Phenylalanine, Glutamate, Aspartate, Glycine,
10 Alanine, Valine, Leucine, Isoleucine, Methionine, Serine, Threonine, Asparagine, Glutamine, Proline and Cysteine, provided that:

when mutated to Arginine, Leucine₅₄ is not mutated to Valine and/or
Leucine₆₄ is not mutated to Arginine;

when mutated to Cysteine, another residue is also mutated;

15 when mutated to Leucine, Histidine₅₁ is not mutated to Tyrosine; and
when mutated to Glycine, Aspartate₃₃ is not mutated to Tyrosine.

18. A modified human β_2m as claimed in claim 17, wherein Tryptophan₆₀ is replaced with Glycine.

20

19. A modified human β_2m in which Lysine₅₈ is replaced with Arginine and Tryptophan₆₀ is replaced with Glycine.

20. A modified human β_2m in which Serine₆₁ is replaced with one or more of
25 Arginine, Histidine, Tryptophan, Tyrosine, Glutamate, Aspartate, Glycine, Alanine, Valine, Leucine, Isoleucine, Methionine, Threonine, Asparagine, Glutamine, Proline and Cysteine.

21. A protein coded for by the nucleic acid sequences as shown in Figure 1a herein, when modified as shown in any one of Figures 1b-1l.
22. A protein which is a homologue or derivative of the protein as claimed in any
5 one of claims 12 to 21.
23. A modified β_2 -microglobulin whose binding to CD8 is inhibited, or a protein as claimed in any one of claims 12 to 22, for use in medicine.
- 10 24. The use of a modified β_2 -microglobulin whose binding to CD8 is inhibited, or a protein as claimed in any one of claims 12 to 22, in the manufacture of a medicament for inhibiting CD8⁺ T cell response.
25. The use as claimed in claim 24, modified by the features of any one of claims
15 2 to 11.
26. A method of inhibiting the binding of a CD8⁺ T cell to a class I Major Histocompatibility Complex (MHC), the method comprising exposing the class I MHC to a modified β_2 -microglobulin whose binding to CD8 is inhibited, or a protein
20 as claimed in any one of claims 12 to 22.
27. A nucleic acid molecule comprising or consisting of a sequence which is:
- (i) the DNA sequence set out in Figure 1a herein, when modified as shown in any one of Figures 1b-1l, or its RNA equivalent;
 - 25 (ii) a sequence which is complementary to the sequence of (i);
 - (iii) a sequence which codes for the same protein or polypeptide, as the sequence of (i) or (ii);

(iv) a sequence which has substantial identity with any of those of (i), (ii) and (iii);

(v) a sequence which codes for a homologue, derivative or fragment of a protein as defined in Figure 1a, when modified as shown in any one of Figures 1b-1l.

5

28. A vector comprising the nucleic acid molecule as claimed in claim 27.

29. A host cell including the vector as claimed in claim 28.

1/19

Figure 1a

atcATCCAGCGTACTCCAAAGATTcAGGTTTACTCACGTCATCCAGCAGAGAATGGAAAGTCAAATTTCTCTGA Base
 tacTAGGTCGCATGAGGTTTCTAAGTCAAATGAGTGCAGTAGGTCGTCTCTTACCTTTCAGTTTAAAGGACT 70
 M I Q R T P K I Q V Y S R H P A E N G K S N F L N
 1 10 20

ATTGCTATGTGTCTGGGTTTCATCCATCCGACATTGAAGTTGACTTACTGAAGAATGGAGAGAGAATTGA Base
 TAACGATACACAGACCCAAAGTAGGTAGGCTGTAACCTCAACTGAATGACTTCTTACCTCTCTCTTAAC 140
 C Y V S G F H P S D I E V D L L K N G E R I E
 30 40

AAAAGTGGAGCATTcAGACTTGTCTTTcAGCAAGGACTGGTCTTTCTATCTCTTGTACTACACTGAATTC Base
 TTTTCACCTCGTAAGTCTGAACAGAAAGTCGTTCCTGACCAGAAAGATAGAGAACATGATGTGACTTAAG 210
 K V E H S D L S F S K D W S F Y L L Y Y T E F
 50 60 70

ACCCCACTGAAAAAGATGAGTATGCCTGCCGTGTGAACCATGTGACTTTGTACAGCCCAAGATAGTTA Base
 TGGGGGTGACTTTTTCTACTCATACGGACGGCACACTTGGTACACTGAAACAGTGTGGGTTCTATCAAT 280
 T P T E K D E Y A C R V N H V T L S Q P K I V K
 80 90

AGTGGGATCGAGACATGTAA Base pairs
 TCACCCTAGCTCTGTACATT 281 to 300
 W D R D M *

Figure 1b: K58 → E

Δ NruI
 AAAAGTGGAGCATTcAGACTTGTCTTTcTctgagGACTGGTCTTTCTATCTCTTGTACTACACTGAATTC Base
 TTTTCACCTCGTAAGTCTGAACAGAAAGagactcCTGACCAGAAAGATAGAGAACATGATGTGACTTAAG 210
 K V E H S D L S F S E D W S F Y L L Y Y T E F
 50 60 70

Figure 1c: K58 → S

Δ NruI
 AAAAGTGGAGCATTcAGACTTGTCTTTcTcttctGACTGGTCTTTCTATCTCTTGTACTACACTGAATTC Base
 TTTTCACCTCGTAAGTCTGAACAGAAAGagaagaCTGACCAGAAAGATAGAGAACATGATGTGACTTAAG 210
 K V E H S D L S F S S D W S F Y L L Y Y T E F
 50 60 70

Figure 1d: K58 → R

Δ NruI
 AAAAGTGGAGCATTcAGACTTGTCTTTcTctcgcGACTGGTCTTTCTATCTCTTGTACTACACTGAATTC Base
 TTTTCACCTCGTAAGTCTGAACAGAAAGagagcgcCTGACCAGAAAGATAGAGAACATGATGTGACTTAAG 210
 K V E H S D L S F S R D W S F Y L L Y Y T E F
 50 60 70

Figure 1e: K58 → Y

Δ NruI
 AAAAGTGGAGCATTcAGACTTGTCTTTcTcttacGACTGGTCTTTCTATCTCTTGTACTACACTGAATTC Base
 TTTTCACCTCGTAAGTCTGAACAGAAAGagaatgCTGACCAGAAAGATAGAGAACATGATGTGACTTAAG 210
 K V E H S D L S F S Y D W S F Y L L Y Y T E F
 50 60 70

2/19

Figure 1f: K58 → C

$\Delta NruI$

```

AAAAGTGGAGCATTGAGACTTGTCTTTGAGctgtGACTGGTCTTTCTATCTCTTGTACTACACTGAATTC  Base
TTTTACCTCGTAAGTCTGAACAGAAAGTCGacactGACCAGAAAGATAGAGAACATGATGTGACTTAAG  210
K V E H S D L S F S C D W S F Y L L Y Y T E F
      50                      60                      70

```

Figure 1g: K58 → SES

```

AAAAGTGGAGCATTGAGACTTGTCTTTGAGctgtgagtctGACTGGTCTTTCTATCTCTTGTACTACACTGAATTC  Base
TTTTACCTCGTAAGTCTGAACAGAAAGTCGagactcgaactGACCAGAAAGATAGAGAACATGATGTGACTTAAG  210
K V E H S D L S F S S E S D W S F Y L L Y Y T E F

```

Figure 1h: K58 → W

```

AAAAGTGGAGCATTGAGACTTGTCTTTCTCT tggGACTGGTCTTTCTATCTCTTGTACTACACTGAATTC  Base
TTTTACCTCGTAAGTCTGAACAGAAAGAGAAccCTGACCAGAAAGATAGAGAACATGATGTGACTTAAG  210
K V E H S D L S F S W D W S F Y L L Y Y T E F

```

Figure 1i: K58 → GRG

```

AAAAGTGGAGCATTGAGACTTGTCTTTCTCTggtcgcggcGACTGGTCTTTCTATCTCTTGTACTACACTGAATTC  Base
TTTTACCTCGTAAGTCTGAACAGAAAGAGAccagcgcgCTGACCAGAAAGATAGAGAACATGATGTGACTTAAG  210
K V E H S D L S F S G R G D W S F Y L L Y Y T E F

```

Figure 1j: D59 → GEG

```

AAAAGTGGAGCATTGAGACTTGTCTTTGAGCAAGggtgagggcTGGTCTTTCTATCTCTTGTACTACACTGAATTC  Base
TTTTACCTCGTAAGTCTGAACAGAAAGTCGTTCCcactcccgACCAGAAAGATAGAGAACATGATGTGACTTAAG  210
K V E H S D L S F S K G E G W S F Y L L Y Y T E F

```

Figure 1k: W60 → G

```

AAAAGTGGAGCATTGAGACTTGTCTTTGAGCAAGGACggtTCTTTCTATCTCTTGTACTACACTGAATTC  Base
TTTTACCTCGTAAGTCTGAACAGAAAGTCGTTCCCTGccaAGAAAGATAGAGAACATGATGTGACTTAAG  210
K V E H S D L S F S K D G S F Y L L Y Y T E F

```

Figure 1l: K58W60 → RG

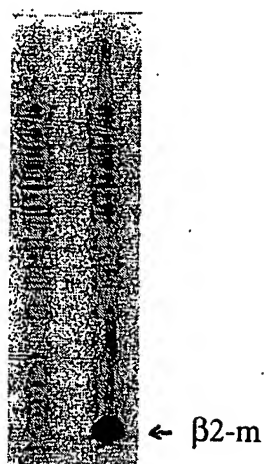
```

AAAAGTGGAGCATTGAGACTTGTCTTTCTCTCGCGACggtTCTTTCTATCTCTTGTACTACACTGAATTC  Base
TTTTACCTCGTAAGTCTGAACAGAAAGAGAGCGCTGccaAGAAAGATAGAGAACATGATGTGACTTAAG  210
K V E H S D L S F S R D G S F Y L L Y Y T E F

```

3/19

FIGURE 2



4/19

FIGURE 3

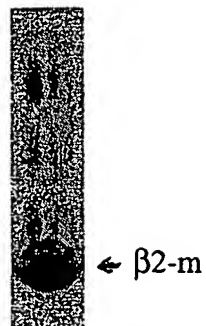
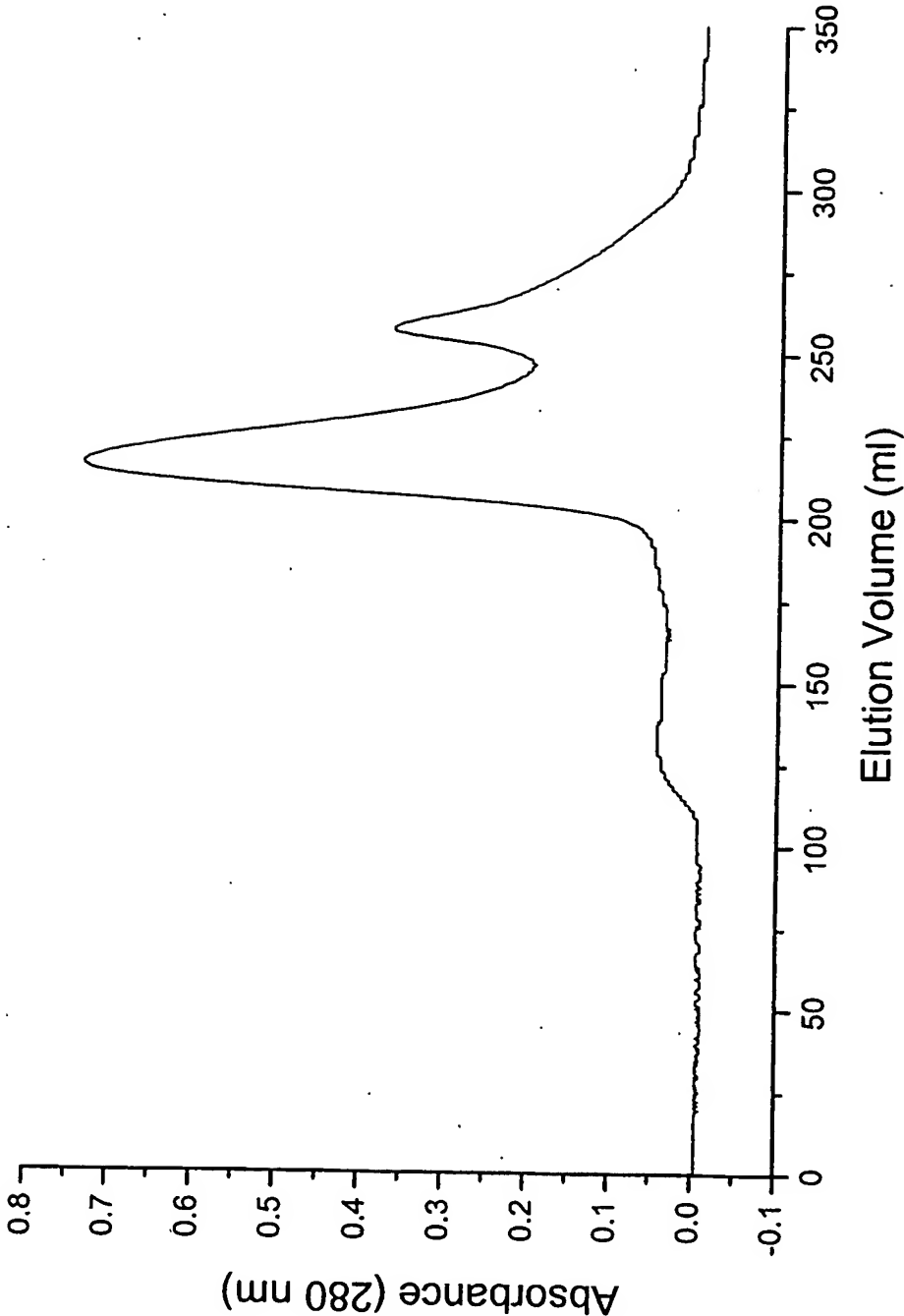


FIGURE 4




6/19

FIGURE 5



FIGURE 6

7/19

Peptide antigen 1 (class I HLA presented) Soluble immobilised HLA molecule 1 (class I type). The β 2-microglobulin subunit is illustrated as the black square

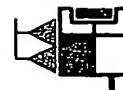
Soluble immobilised HLA molecule 1 (class I) loaded with peptide antigen



Soluble CD8 receptor (for class I HLA binding), unbound



Soluble CD8 receptor bound to HLA class I molecule



Soluble T cell receptor (specific for Peptide Antigen 1 presented by class I HLA), unbound



Soluble T cell receptor (specific for Peptide Antigen 1 presented by class I HLA). T cell receptor bound to HLA/peptide

Soluble immobilised HLA molecule 1 with a mutation (mutation 1) in the β 2-microglobulin subunit that prevents, or significantly inhibits, CD8 bindingSoluble immobilised HLA molecule 1 with a mutation (mutation 2) in the β 2-microglobulin subunit that prevents, or significantly inhibits, CD8 bindingSoluble immobilised HLA molecule 1 with a mutation (mutation 3) in the β 2-microglobulin subunit that does not prevent, or significantly inhibit, CD8 binding

Biosensor readout, no binding to HLA molecule

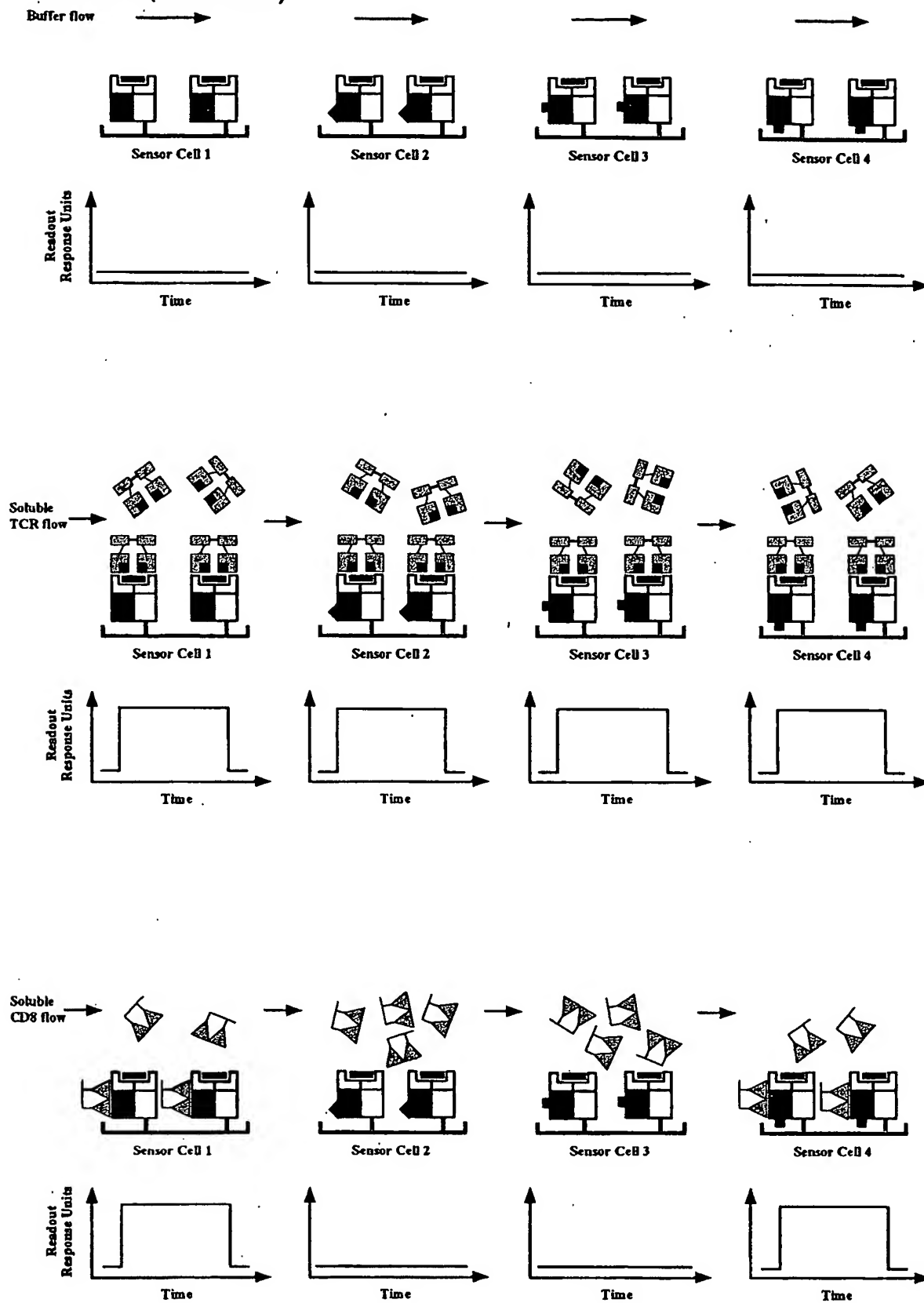


Biosensor readout, transient binding of large molecule (TCR or CD8 receptor) to HLA molecule



8/19

FIGURE 6 (continued)



9/19

Figure 7a

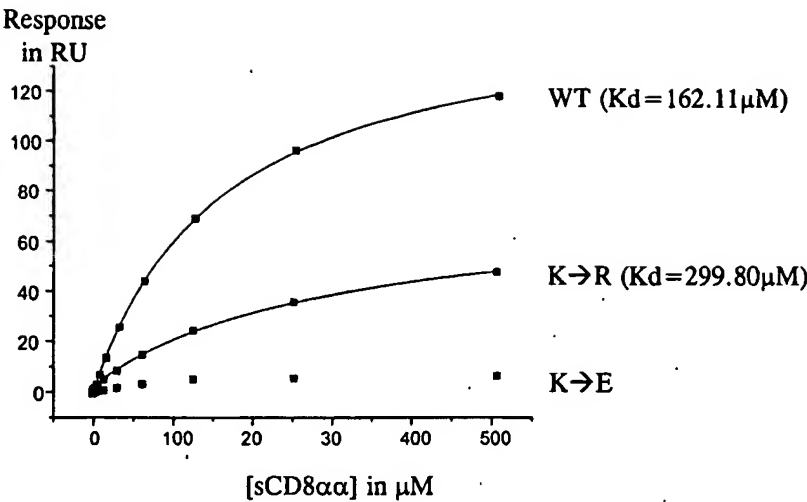


Figure 7b

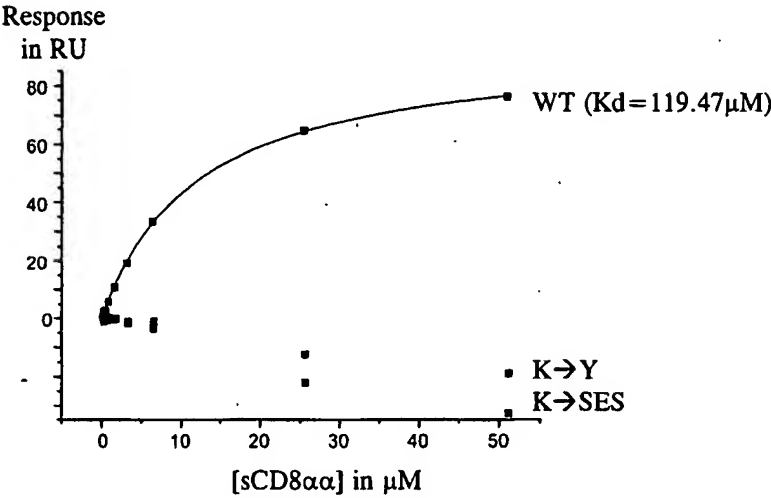
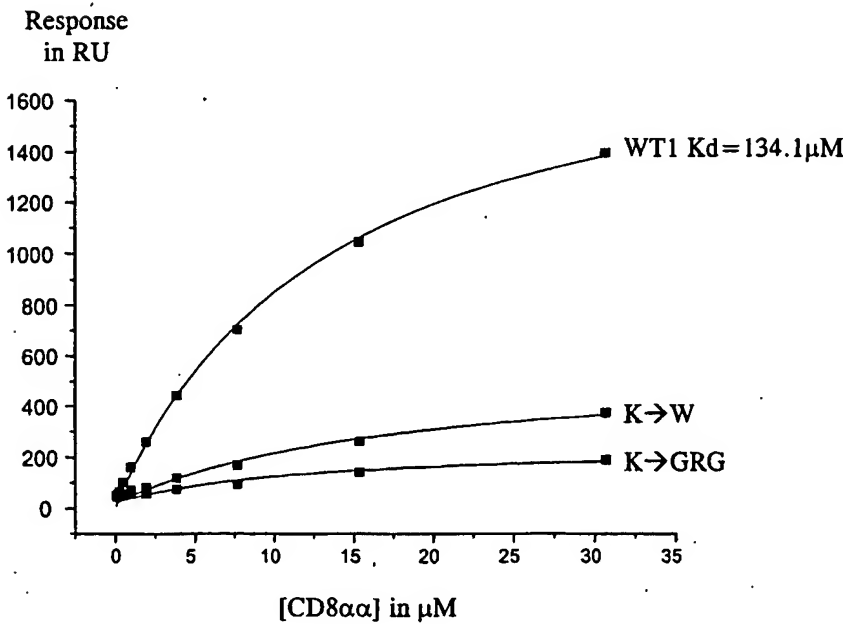


Figure 7c



10/19

Figure 7d

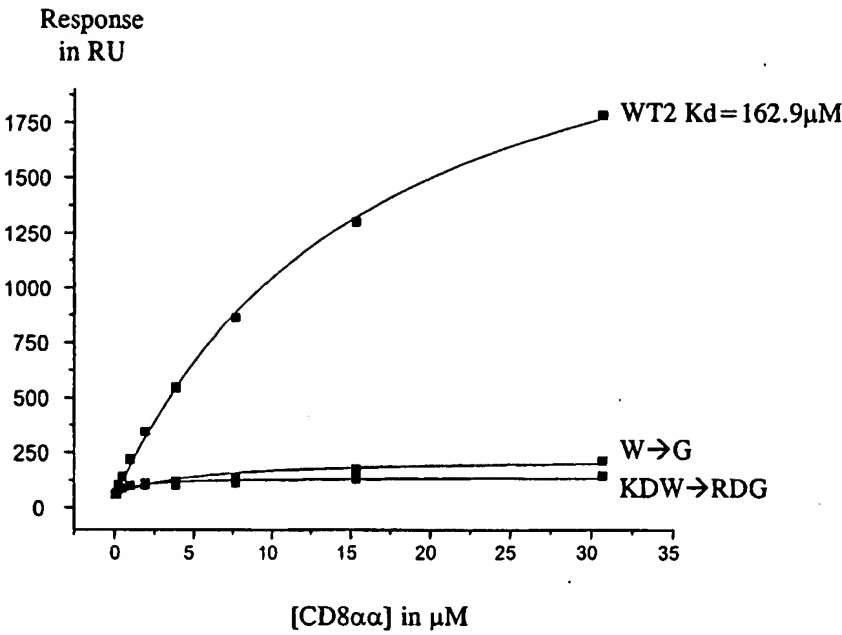


Figure 7e

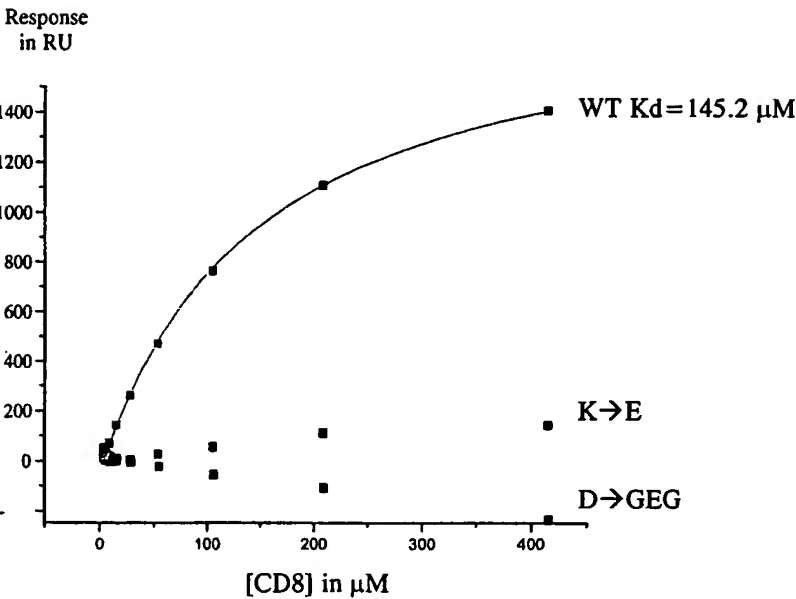


Figure 8a

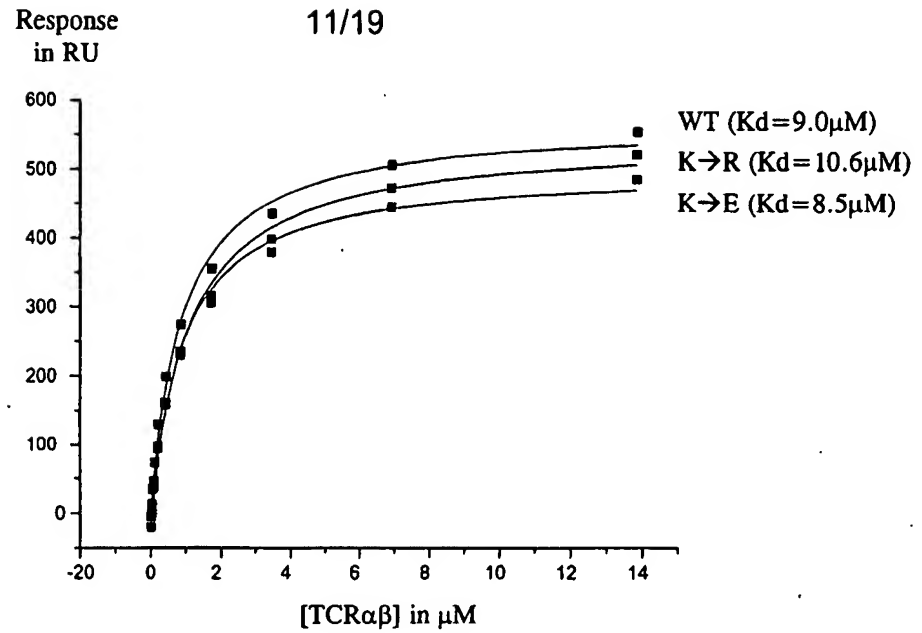


Figure 8b

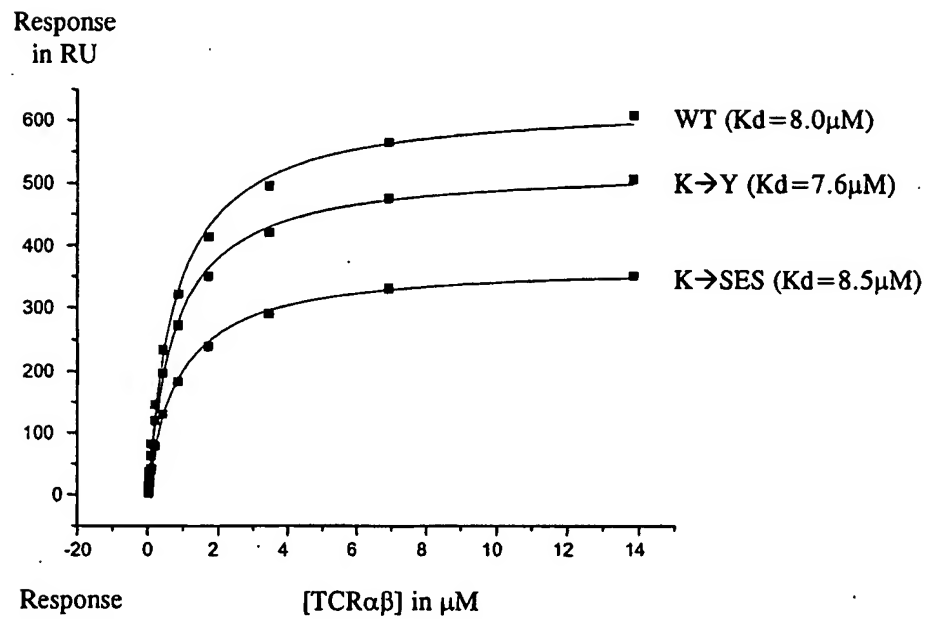


Figure 8c

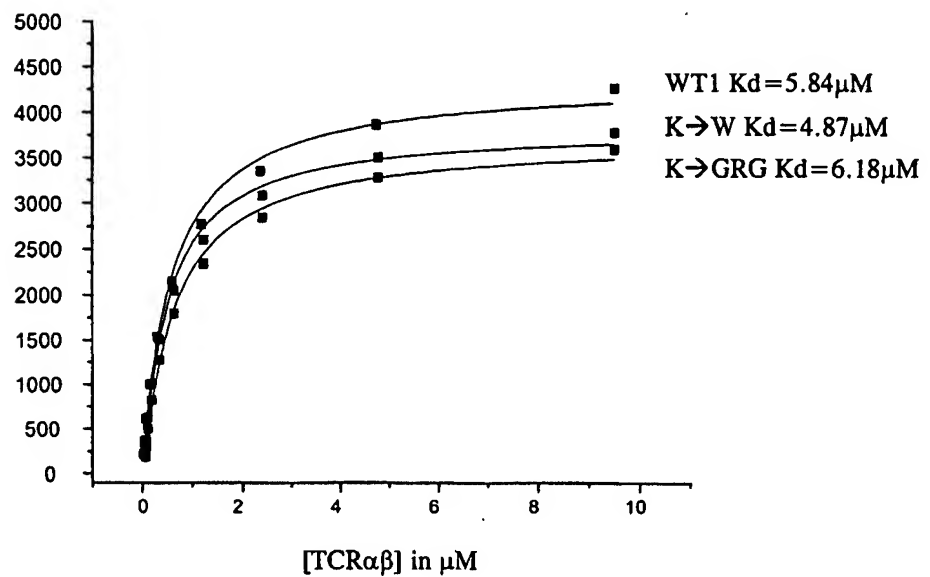


Figure 8d

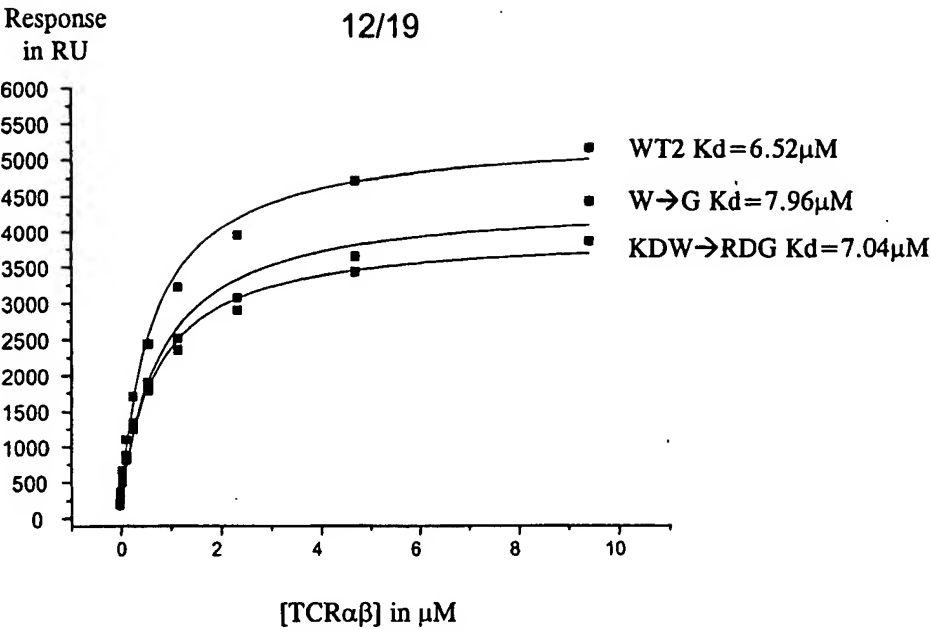


Figure 8e

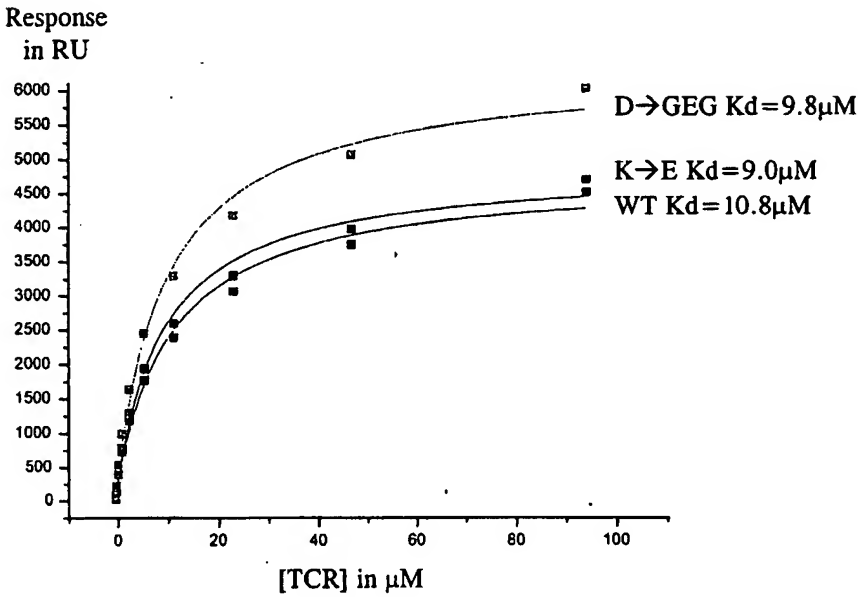


Figure 9a

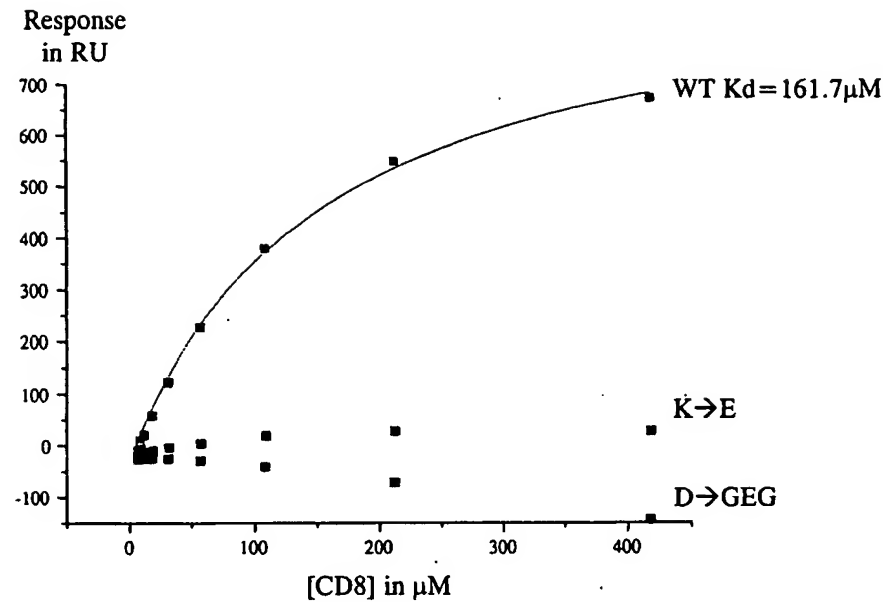


Figure 9b

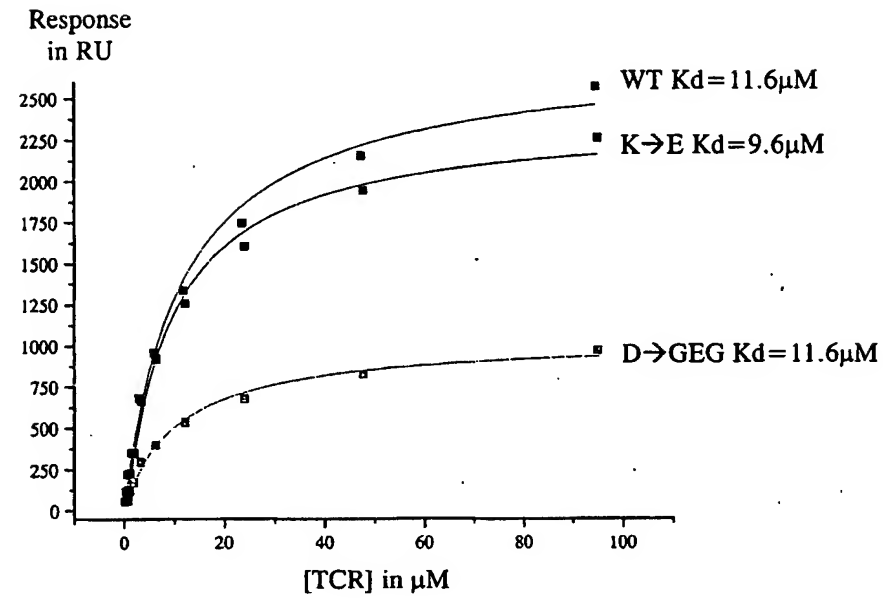


Figure 10

14/19

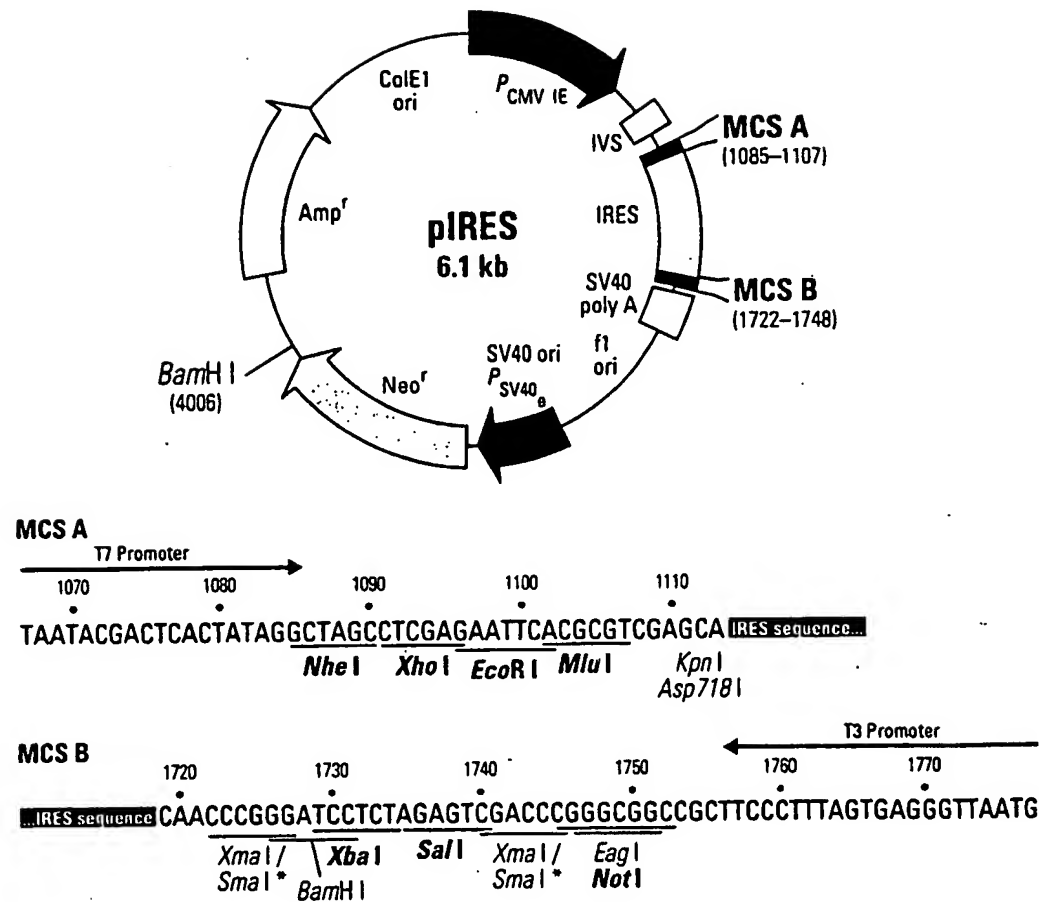


Figure 11

1098 base pairs

ATGCGCGTCATGGCGCCCCGAACCCTCGTCCTGCTACTCTCGGGGGCTCTGGCCCTGACCCAGACCTGGG	Base pairs
TACCGGCAGTACCGCGGGGCTTGGGAGCAGGACGATGAGAGCCCCGAGACCGGGACTGGGTCTGGACCC	1 to 70
CGGGCTCTCACTCCATGAGGTATTTCTTCACATCCGTGTCCCGGGCCGGCCGGGAGCCCCGCTTCAT	Base pairs
GCCCCGAGAGTGAGGTACTCCATAAAGAAGTGTAGGCACAGGGCCGGCCGGCGCCCCCTCGGGGCGAAGTA	71 to 140
CGCAGTGGGCTACGTGGACGACACGCAGTTCTGTGCGGTTCTGACAGCGACGCCGCGAGCCAGAGGATGGAG	Base pairs
GCGTCACCCGATGCACCTGCTGTGCGTCAAGCACGCCAAGCTGTCGCTGCGGCGCTCGGTCTCCTACCTC	141 to 210
CCGCGGGCGCCGTGGATAGAGCAGGAGGTCCGGAGTATTGGGACGGGGAGACACGAAAGTGAAGGCC	Base pairs
GGCGCCCGCGGCACCTATCTCGTCCTCCAGGCCTCATAACCTGCCCCCTCTGTGCCTTTCACTTCCGGG	211 to 280

15/19

Figure 1:1 contd

ACTCACAGACTCACCGAGTGGACCTGGGGACCTGCGCGGCTACTACAACCAGAGCGAGGCCGGTTCTCA Base pairs
 TGAGTGTCTGAGTGGCTCACCTGGACCCCTGGGACGCGCGGATGATGTTGGTCTCGCTCCGGCCAAGAGT 281 to 350

CACCGTCCAGAGGATGTATGGCTGCGACGTGGGGTCGGACTGGCGCTTCTCCGCGGTACCACCAGTAC Base pairs
 GTGGCAGGTCTCTACATACCGACGCTGCACCCAGCCTGACCGCGAAGGAGGCGCCCATGGTGGTCATG 351 to 420

GCCTACGACGGCAAGGATTACATCGCCCTGAAAGAGGACCTGCGCTCTTGGACCGCGCGGACATGGCAG Base pairs
 CGGATGCTGCCGTTCCTAATGTAGCGGACTTTCTCCTGGACGCGAGAACCTGGCGCCGCTGTACCGTC 421 to 490

CTCAGACCACCAAGCACAGTGGGAGGCGGCCCATGTGGCGGAGCAGTTGAGAGCCTACCTGGAGGGCAC Base pairs
 GAGTCTGGTGGTTCGTGTTACCCCTCCGCCGGGTACACCGCCTCGTCAACTCTCGGATGGACCTCCCGTG 491 to 560

GTGCGTGGAGTGGCTCCGCAGATACCTGGAGAACGGGAAGGAGACGCTGCAGCGCACGGACGCCCCAAA Base pairs
 CACGCACCTCACCGAGGCGTCTATGGACCTCTTGCCCTTCCTCTGCGACGTCGCGTGCTGCGGGGGTTT 561 to 630

ACGCATATGACTCACCACGCTGTCTCTGACCATGAAGCCACCCTGAGGTGCTGGGCCCTGAGCTTCTACC Base pairs
 TCGGTATACTGAGTGGTGCACAGAGACTGGTACTTCGGTGGGACTCCACGACCCGGGACTCGAAGATGG 631 to 700

CTGCGGAGATCACACTGACCTGGCAGCGGGATGGGGAGGACCAGACCCAGGACACGGAGCTCGTGGAGAC Base pairs
 GACGCCTCTAGTGTGACTGGACCGTCGCCCTACCCCTCCTGGTCTGGGTCTGTGCCTCGAGCACCTCTG 701 to 770

CAGGCCTGCAGGGGATGGAACCTTCCAGAAGTGGGCGGCTGTGGTGGTGCCTTCTGGACAGGAGCAGAGA Base pairs
 GTCCGGACGTCCCCTACCTTGAAGGTCTTACCCGCGGACACCACCGGAAGACCTGTCTCTCGTCTCT 771 to 840

TACACCTGCCATGTGCAGCATGAGGGTTTGCCCAAGCCCTCACCCCTGAGATGGGAGCCGTCTTCCCAGC Base pairs
 ATGTGGACGGTACACGTCGTACTCCCAAACGGGTTGCGGGAGTGGGACTCTACCCCTCGGCAGAAGGGTCG 841 to 910

CCACCATCCCCATCGTGGGCATCATTGCTGGCCTGGTTCTCTTTGGAGCTGTGATCACTGGAGCTGTGGT Base pairs
 GGTGGTAGGGGTAGCACCCGTAGTAACGACCGGACCAAGAGAAACCTCGACACTAGTGACCTCGACACCA 911 to 980

CGCTGCTGTGATGTGGAGGAGGAAGAGCTCAGATAGAAAAGGAGGGAGCTACTCTCAGGCTGCAAGCAGT Base pairs
 GCGACGACACTACACCTCCTCCTCTCGAGTCTATCTTTCCCTCCCTCGATGAGAGTCCGACGTTCTGTA 981 to 1050

GACAGTGCCCGGGCTCTGATGTGTCTCTCACAGCTTGTAAGTGTGA Base pairs
 CTGTACCGGTCGCCGAGACTACACAGAGAGTGTGGAACATTTCACT 1051 to 1098

A2-F1: aaaccggtctagaGGATGGCCGTCATGGCGCC

A2-R1: cccgpgccgcTCACACTTTACAAGCTGTGAGAGAC

16/19

Figure 12

ATGTCTCGCTCCGTGGCCTTAGCTGTGCTCGCGCTACTCTCTCTTTCTGGCCTGGAGGCTATCCAGCGTA Base pairs
TACAGAGCGAGGCACCGGAATCGACACGAGCGCGATGAGAGAGAAAGACCGGACCTCCGATAGGTGCGAT 1 to 70

CTCCAAAGATTACGTTTACTCACGTATCCAGCAGAGAATGGAAAGTCAAATTCCTGAATTGCTATGT Base pairs
GAGGTTTCTAAGTCCAAATGAGTGCAGTAGGTCGTCTCTTACCTTTCAGTTTAAAGGACTTAACGATACA 71 to 140

GTCTGGGTTTCATCCATCCGACATTGAAGTTGACTTACTGAAGAATGGAGAGAGAATTGAAAAAGTGGAG Base pairs
CAGACCCAAAGTAGGTAGGCTGTAACCTCAACTGAATGACTTCTTACCTCTCTCTTAACCTTTTTCACCTC 141 to 210

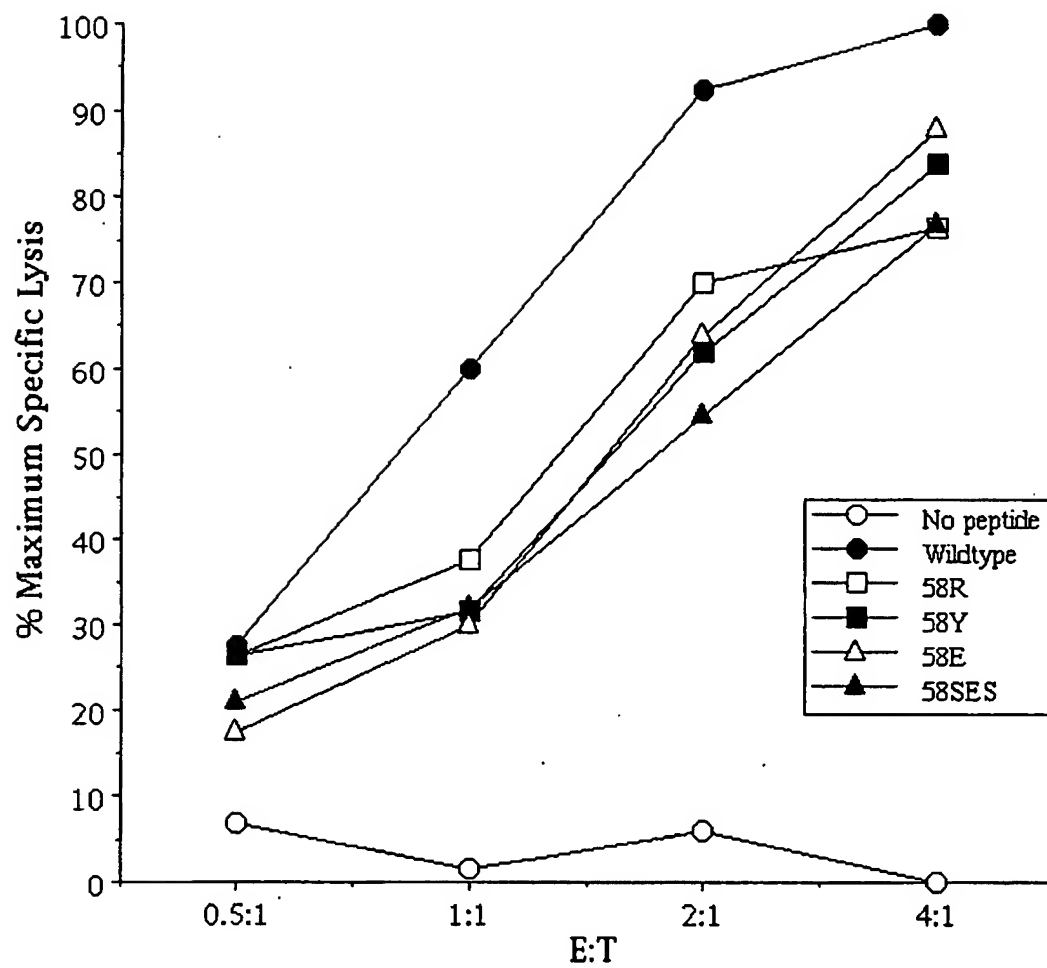
CATTCAGACTTGTCTTTTCAGCAAGGACTGGTCTTTCTATCTCTTGTACTTAACTGAATTCACCCCACTG Base pairs
GTAAGTCTGAACAGAAAGTCGTTCTGACCAGAAAGATAGAGAACATGATGTGACTTAAGTGGGGGTGAC 211 to 280

AAAAAGATGAGTATGCCTGCCGTGTGAACCATGTGACTTTGTACAGCCCAAGATAGTTAAGTGGGATCG Base pairs
TTTTTCTACTCATACGGACGGCACACTTGGTACACTGAAACAGTGTGGGTTCTATCAATTCACCCTAGC 281 to 350

AGACATGTAA Base pairs

TCTGTACATT 351 to 360

17/19



18/19

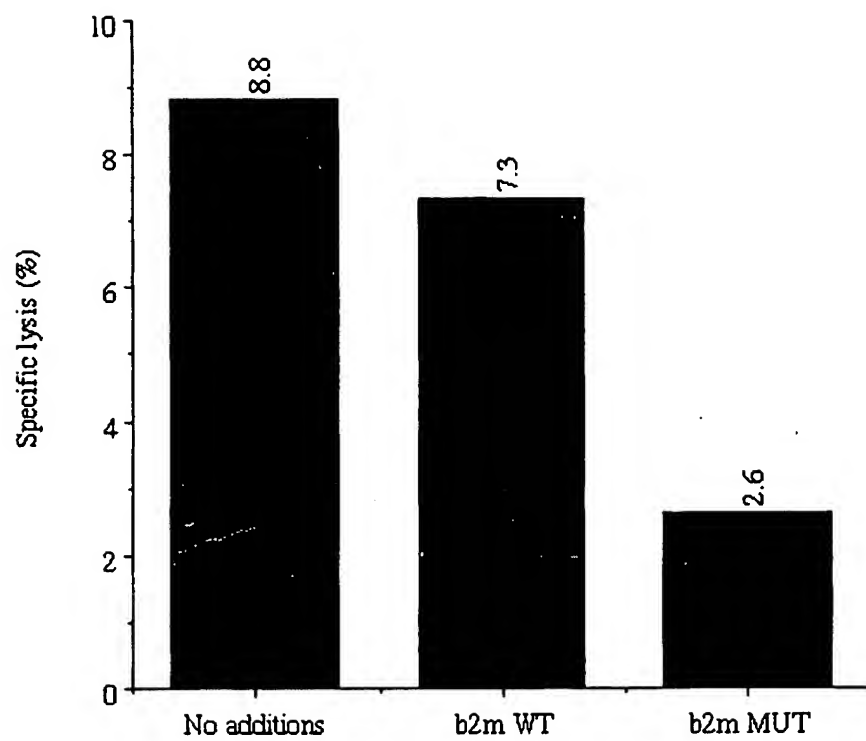


Figure 14

19/19

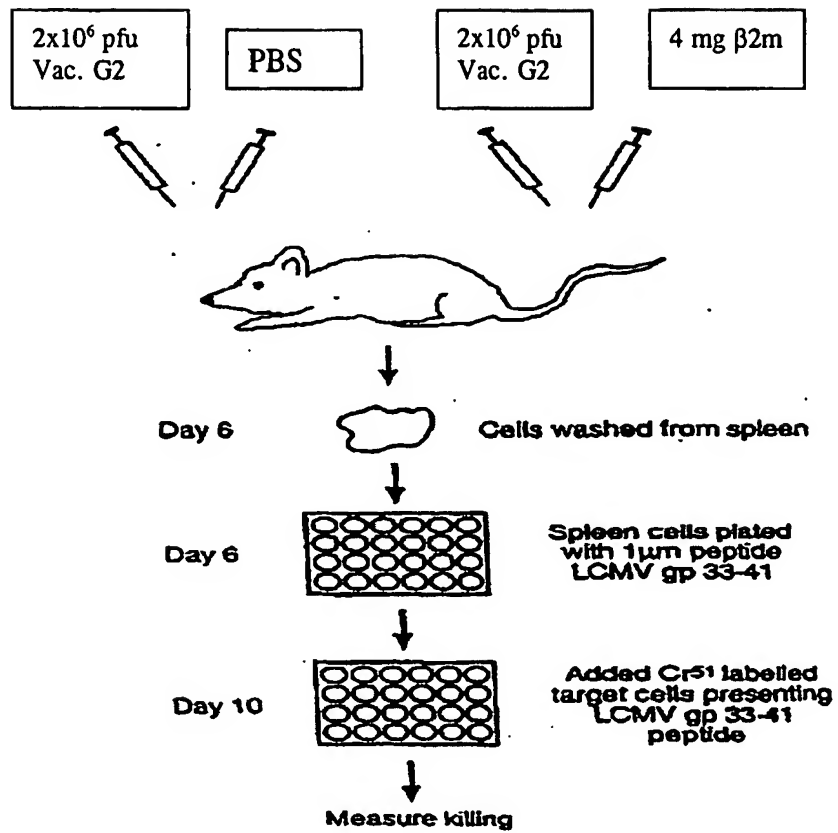


Figure 15

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07K14/705

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal, WPI Data, PAJ, MEDLINE, CHEM ABS Data, INSPEC

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VAN EYNDHOVEN WINIFRED G ET AL: "Changes in rheumatoid factor and monoclonal IgG antibody specificity after site-specific mutations in antigenic region of beta-2-microglobulin." CLINICAL IMMUNOLOGY AND IMMUNOPATHOLOGY, vol. 72, no. 3, 1994, pages 362-372, XP000995144 ISSN: 0090-1229	12-23, 27-29
Y	abstract; figures 1-4; tables 1-5 --- -/--	1-11, 24-26

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- * & * document member of the same patent family

Date of the actual completion of the international search

19 April 2001

Date of mailing of the international search report

03/05/2001

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
 NL - 2280 HV Rijswijk
 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
 Fax: (+31-70) 340-3016

Authorized officer

Novak, S

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WILLIAMS RALPH C JR ET AL: "Antigenic epitopes on beta-2-microglobulin reacting with monoclonal antibodies." JOURNAL OF LABORATORY AND CLINICAL MEDICINE, vol. 121, no. 6, 1993, pages 805-820, XP000995194 ISSN: 0022-2143	12-23, 27-29
Y	abstract; figures 1,4; tables II,III	1-11, 24-26
X	----- FUJIURA YASUYOSHI ET AL: "Development of CD8-alpha-alpha+ intestinal intraepithelial T cells in beta-2-microglobulin- and/or TAP1-deficient mice." JOURNAL OF IMMUNOLOGY, vol. 156, no. 8, 1996, pages 2710-2715, XP000995185 ISSN: 0022-1767	1-4, 26
Y	the whole document	5-25, 27-29
Y	----- GAO ET AL: "Assembly and crystallization of the complex between the human T cell coreceptor CD8alpha homodimer and HLA-A2" PROTEIN SCIENCE, GB, CAMBRIDGE UNIVERSITY PRESS, CAMBRIDGE, vol. 7, no. 5, 1 May 1998 (1998-05-01), pages 1245-1249, XP002093586 ISSN: 0961-8368 the whole document	1-29
Y	----- WO 99 21576 A (GERTH ULRICH CONRAD ; JAKOBSEN BENT KARSTEN (GB); GAO GEORGE FU (GB) 6 May 1999 (1999-05-06) page 3, line 22 -page 13, line 14	1-29
Y	----- GAO G F ET AL: "Crystal structure of the complex between human CD8alpha(alpha) and HLA-A2" NATURE, GB, MACMILLAN JOURNALS LTD. LONDON, vol. 387, no. 6633, 5 June 1997 (1997-06-05), pages 630-634, XP002093584 ISSN: 0028-0836 the whole document	1-29
	----- -/--	

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SERREZE D V ET AL: "MAJOR HISTOCOMPATIBILITY COMPLEX CLASS I-DEFICIENT NOD-B2MNULL MICEARE DIABETES AND INSULITIS RESISTANT" DIABETES,US,NEW YORK, NY, vol. 43, no. 3, 1 March 1994 (1994-03-01), pages 505-509, XP000603733 ISSN: 0012-1797 ---	
A	US 5 051 371 A (LARSEN FLEMMING S ET AL) 24 September 1991 (1991-09-24) ---	
A	UGER ET AL: "Creating CTL targets with epitope-linked beta 2-microglobulin constructs" JOURNAL OF IMMUNOLOGY,US,THE WILLIAMS AND WILKINS CO. BALTIMORE, vol. 160, no. 4, 15 February 1998 (1998-02-15), pages 1598-1605, XP002115504 ISSN: 0022-1767 ---	
A	WO 99 64597 A (RIBAUDO RANDALL K ;US HEALTH (US); SHIELDS MICHAEL (US)) 16 December 1999 (1999-12-16) ---	
A	PRINCE H E ET AL: "INTERRELATIONSHIPS BETWEEN SEROLOGIC MARKERS OF IMMUNE ACTIVATION AND T LYMPHOCYTE SUBSETS IN HIV INFECTION" JOURNAL OF ACQUIRED IMMUNE DEFICIENCY SYNDROMES, vol. 3, no. 5, 1990, pages 525-530, XP000995130 ISSN: 0894-9255 ---	
A	US 5 674 681 A (ROTHENBERG BARRY E) 7 October 1997 (1997-10-07) -----	

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9921576	A	06-05-1999	AU 9637498 A	17-05-1999
			CN 1283120 T	07-02-2001
			EP 1024822 A	09-08-2000
			NO 20002148 A	27-06-2000
<hr/>				
US 5051371	A	24-09-1991	DK 21586 A	14-09-1987
			AU 601963 B	27-09-1990
			AU 6763987 A	23-07-1987
			EP 0229723 A	22-07-1987
			FI 870160 A	17-07-1987
			JP 62246596 A	27-10-1987
			NO 870174 A	17-07-1987
			PT 84126 A, B	01-02-1987
			US 5175113 A	29-12-1992
<hr/>				
WO 9964597	A	16-12-1999	AU 4545799 A	30-12-1999
			EP 1086224 A	28-03-2001
<hr/>				
US 5674681	A	07-10-1997	AU 711726 B	21-10-1999
			AU 4466296 A	26-06-1996
			CA 2207103 A	13-06-1996
			CN 1173184 A	11-02-1998
			EP 0820469 A	28-01-1998
			JP 10510424 T	13-10-1998
			WO 9617870 A	13-06-1996
<hr/>				